PSC-derived neuronal co-cultures to model corticostriatal connectivity loss and associated phenotypes observed in Huntington's disease

Caroline Casey

Prof Sarah Tabrizi & Dr Selina Wray

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Declaration

I, Caroline Casey confirm that the work presented in this thesis is my own. Where information has been derived from other sources or the contributions of others have been involved, this has been clearly indicated in the text.

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Abstract
Huntington's disease (HD) is a genetic neurodegenerative disease caused by an expanded CAG-repeat mutation in exon 1 of the gene encoding huntingtin (HTT) which results in an elongated polyglutamine (polyQ) tract. A triad of symptoms encompassing cognitive, motor and psychiatric features are evident in mutant gene carriers, with typical symptom manifestation in adult life; the degeneration of the corticostriatal (CS) pathway and disruption to its connectivity partially underlie the manifestation of these symptoms. The CS pathway is composed of cortical layer V projection neurons (CPNs) and striatal medium spiny neurons (MSNs), which are the principle neuronal subtypes targeted in HD pathology. The aim of this thesis was to recapitulate the CS pathway in vitro using human cells in co-culture, in order to better understand the early pathological events that lead to its disruption in HD. Using human pluripotent stem cells (PSC) derived from an HD family and an isogenic HTT allelic series, MSN- and CPN-containing cultures expressing various HTT polyQ lengths were generated using validated methods that were then adapted and optimised for use in this thesis.

HD-associated phenotypes were revealed in both cell types by using a thorough approach to phenotypical profiling. Alterations in cell viability at baseline and after stress were documented, as well as changes to neuronal morphology and adhesive function. Novel investigations into axonal projection revealed a HTT polyQ-length dependent deficiency in HD CPNs, as well as altered neurotrophin production and release. Using microfluidic chambers (MFC), a co-culture system was created to recapitulate the CS pathway. Their use showed that it is possible to culture PSC-derived neurons in MFCs, with evidence of neuronal connectivity as shown by the formation of synapses within the devices. Differences in the level of synapse maintenance suggest that this platform could be useful in further assessing the development of early CS pathology in an HD context.
Impact statement

This thesis is the first known example of modelling the corticostriatal (CS) pathway with human neurons in vitro. The CS pathway is key in Huntington's disease pathogenesis, a neurodegenerative disease that is caused by a genetic mutation for which there are currently no disease modifying therapies. The importance of studying HD in a human context is evident due to the wider discrepancy between pre-clinical animal studies, and subsequent trials in humans; so far studies that were successful in pre-clinical stages have failed to meet their targets when tested clinically. The assumption here is that the discrepancy in results is due to the different species, thus generating the CS pathway using human neurons is of huge impact. It is also possible that limitations in trial design may contribute to this lack of clinical effect however.

The best technology at present to bridge the gap between pre-clinical and clinical studies, is pluripotent stem cell (PSC)-technology. This thesis presents the rigorous characterisation of human PSC-derived striatal medium spiny neurons (MSNs) and cortical layer V projection neurons (CPNs) that make up the CS pathway in the brain. Detailed are the methodologies used to investigate novel aspects of HD pathiology, with results that suggest specific phenotypes are influenced in their severity by the length of disease-causing HTT CAG-repeat expansion. These methods are in preparation for publication and will be available for use by the rest of the HD research community as well as the field of neurodegenerative disease as a whole.

Similarly, details of the optimisation, generation and characterisation of the microfluidic chamber (MFC) platform in which CS co-cultures were generated will also be available. This technique has not been used before with human HD PSC-derived neurons, thus it provides a novel platform on which future studies can be built. The phenotypes recorded from CS co-cultures in MFCs highlight how the model species used in research may impact results, emphasising further the need for more human-based research in HD.

The main findings of this thesis have been disseminated to both academic and non-academic audiences throughout the past four years, most recently through podium and poster presentations at both the European Huntington's Disease Network, Vienna 2018, and the Gordon: CAG-Triplet Repeat disorders research seminar and conference, Tuscany 2019. Aside, however, from contributing to the knowledge and understanding on HD in the research field, this thesis has the ability to spark interest in the non-academic community. The technique can be viewed in a simplified manner as 'a brain in a dish', a concept of significant interest to a younger generation of potential researchers.
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Abbreviations

< Less than
> More than
± Plus and minus
A2AR Adenosine A2A receptor
AAO Age at onset
AKT Protein kinase B
ALS Amyotrophic lateral sclerosis
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA Analysis of variance
ASO Anti-sense oligonucleotide
BAC Bacterial artificial chromosome
BCA Bicinchoninic acid
BDNF Brain derived neurotrophic factor
BSA Bovine serum albumin
C Celsius
CAG Triplet repeat encoding glutamine
CALB Calbindin
Cas9 CRISPR associated protein 9
Ch Chicken
CNS Central nervous system
CPN Cortical projection neuron
CRISPR Clustered Regularly Interspaced Short Palindromic Repeats
cryo-EM Cryogenic electron microscopy
CS Corticostriatal
CSF Cerebrospinal fluid
CTIP2 COUP-TF-interacting protein 2
d Day
DAMPs Danger-associated molecular patterns
DARPP-32 Dopamine- and cAMP-regulated phosphoprotein, 32 kDa
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DCV</td>
<td>Dense core vesicle</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially expressed genes</td>
</tr>
<tr>
<td>Dk</td>
<td>Donkey</td>
</tr>
<tr>
<td>DLX2</td>
<td>Distal-Less Homeobox 2</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRD</td>
<td>Dopamine receptor</td>
</tr>
<tr>
<td>e.g.</td>
<td>For example</td>
</tr>
<tr>
<td>EAAT2</td>
<td>Excitatory amino acid transporter 2</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENK</td>
<td>Enkephalin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of View</td>
</tr>
<tr>
<td>FOXP2</td>
<td>Forkhead box protein P2</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
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<tr>
<td>GSX2</td>
<td>GS Homeobox 2</td>
</tr>
<tr>
<td>Gt</td>
<td>Goat</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HAP</td>
<td>Huntingtin associated protein</td>
</tr>
<tr>
<td>HARDI</td>
<td>High angular resolution diffusion-weighted data</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HCI</td>
<td>High content imaging</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>Hdh</td>
<td>Mouse huntingtin gene</td>
</tr>
<tr>
<td>HEAT</td>
<td>Huntingtin, elongation factor 3, protein phosphatase 2A, and the lipid kinase TOR</td>
</tr>
<tr>
<td>HTT</td>
<td>Human huntingtin gene</td>
</tr>
<tr>
<td>HTT</td>
<td>Human huntingtin protein</td>
</tr>
<tr>
<td>Htt</td>
<td>Mouse huntingtin protein</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est (that is)</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LN₂</td>
<td>Liquid nitrogen</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>M</td>
<td>Mouse</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEA</td>
<td>Multi-electrode array</td>
</tr>
<tr>
<td>MFC</td>
<td>Microfluidic chamber</td>
</tr>
<tr>
<td>mHTT</td>
<td>Mutant HTT</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>ms</td>
<td>Milliseconds</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neuron</td>
</tr>
<tr>
<td>NANOG</td>
<td>Nanog Homeobox</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
</tr>
<tr>
<td>NRSE</td>
<td>Neuron restrictive silencer element</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>OCT4</td>
<td>octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC1</td>
<td>Prohormone convertase 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE10A</td>
<td>Phosphodiesterase 10A</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-δ-lysine</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PE</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PI3</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PolyQ</td>
<td>Polyglutamine</td>
</tr>
<tr>
<td>PSC</td>
<td>Pluripotent stem cell</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post synaptic density protein 95</td>
</tr>
<tr>
<td>Rb</td>
<td>Rabbit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>Rt</td>
<td>Rat</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SG- II</td>
<td>Secretogranin II</td>
</tr>
<tr>
<td>SIM</td>
<td>Super resolution microscopy</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SOC</td>
<td>Store operated calcium</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>SubP</td>
<td>Substance P</td>
</tr>
<tr>
<td>SYT1</td>
<td>Synaptotagmin I</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>TBR1</td>
<td>T-box, brain, 1</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosine receptor kinase B</td>
</tr>
<tr>
<td>UCL</td>
<td>University College London</td>
</tr>
<tr>
<td>WR</td>
<td>Working reagent</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>Beta-3 tubulin</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
</tbody>
</table>
Chapter 1 – Introduction

1.1 Huntington’s disease

Huntington’s disease (HD) is a hereditary neurodegenerative disease for which the singular cause of pathology has been unequivocally established. First described by Dr George Huntington in 1872, the disease causes involuntary choreic movements and an onset of psychiatric symptoms, which Huntington noted were common to a proportion of individuals within the same family. Indeed it was reported that ‘when either or both the parents have shown manifestations of the disease...one or more of the offspring almost invariably suffer from the disease, if they live to adult age’ (Huntington, 1872), which we now understand to be due to the dominant manner in which the disease is inherited. Extensive investigation has since led to the discovery of the disease-causing gene mutation by the HD research consortium in 1993, which paved the way for better understanding the nature of the disease (MacDonald et al., 1993).

1.1.1 Epidemiology of HD

The prevalence of HD in the Caucasian community is approximately one in 10 000, however this figure is thought to be under reported (Rawlins et al., 2016). Although the disease is typically inherited, de novo mutations may also arise; up to 24% of new HD cases can be attributed to patients with no previous family history of the disease (Almqvist et al., 1999; Myers et al., 1993). In cases where early mortality occurs, or a misdiagnosis is given to elder family members, individuals may be unaware of their HD gene risk status, thus it is anticipated that a higher frequency of the HD mutation is present in the general population (Bates et al., 2015).

Although incidence of HD occurs worldwide (average prevalence is 5.5 per 100 000) (Baig et al., 2016), there are regional pockets with above average prevalence that have attracted specific attention. For example, there is an extremely high prevalence in Venezuela, specifically in a small community near Lake Maracaibo within which 7/1000 residents carry the disease. This community is largely genetically isolated as it is extremely remote, as such intra-familial marriages are common. Based on these huge dynasties, genetic linkage studies pioneered by Gusella et al., in 1983, enabled researchers to identify the specific chromosome on which the Huntington mutation resides.

1.1.2 Genetics of HD

HD gene carriers possess an expansion of a triplet repeat sequence (CAG) within the huntingtin gene (HTT) on chromosome four (4p16.3) (MacDonald et al. 1993). The CAG expansion results in an extended polyglutamine (polyQ) tract within the N terminal region of the huntingtin protein (HTT) (Bates et al., 2015). The number of CAG repeats found within the HTT allele varies both within individuals (resulting in heterozygosity between their two alleles) and within the population, due to the inherent instability of this CAG repeat region.

As described in Table 1, when the number of CAG repeats is <36 the individual is unaffected as the HTT is thought to be fully functional (Ross and Tabrizi, 2011). There have been reports however of ‘intermediate’ gene carriers, who possess 27-35 repeats, and exhibit behavioural
phenotypes that are common also in the HD community, such as apathy and suicidal ideals (Killoran et al., 2013) suggesting a mild phenotype distinct from HD may present in individuals with intermediate allele lengths. For CAG-repeat lengths between 36 and 39, there is incomplete penetrance of the disease, whereby the individual risks developing HD typically much later in life, however this is not guaranteed (Huang et al., 2016). Finally, if CAG length is >39, this is considered an expanded allele, and gene carriers will ultimately develop HD, manifesting symptoms at some point in their lifetime due to a toxic gain of function of the mutant HTT protein produced (Buren et al., 2016a), as well as potential RNA-mediated toxicity.

Table 1. Relationship between CAG repeat length and disease classification. HD = Huntington's disease, AAO = age at onset.

<table>
<thead>
<tr>
<th>CAG-repeat length</th>
<th>Classification</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 27</td>
<td>Unaffected</td>
<td>N/A</td>
<td>Ross and Tabrizi, 2011</td>
</tr>
<tr>
<td>27-35</td>
<td>Intermediate allele length</td>
<td>Mild behavioural phenotype</td>
<td>Killoran et al., 2013</td>
</tr>
<tr>
<td>36-39</td>
<td>Incomplete penetrance</td>
<td>Later AAO for symptom manifestation, disease development not guaranteed</td>
<td>Huang, Chen and Zhang, 2016</td>
</tr>
<tr>
<td>39+</td>
<td>Fully penetrant HD</td>
<td>AAO typically between 35 - 45 years, mortality within 15 years, variable disease severity, hyperkinetic motor phenotype.</td>
<td>Buren et al., 2016</td>
</tr>
<tr>
<td>50+</td>
<td>Juvenile HD</td>
<td>AAO &lt; 20 years of age. Severe phenotype, faster disease progression, bradykinetic motor phenotype</td>
<td>Bates et al., 2015; Cowan and Raymond, 2006</td>
</tr>
</tbody>
</table>

The disease causing mutation is inherited in an autosomal dominant manner; an individual will inherit and develop HD if either of their alleles posses an expanded CAG repeat. For this reason, there is a 50% chance each individual offspring of an affected parent will inherit the disease, and is one of the reasons why HD is one of the more common genetic neurodegenerative diseases (Rawlins et al., 2016).

Within HD families, one common phenomenon is that offspring that have inherited a disease causing allele typically manifest symptoms at an earlier age than their affected parent, and in some cases have a more severe and rapid disease progression (Bates et al., 2015; Myers, 2004; Reiner et al., 2011). This is an example of 'anticipation' and is most common via paternal inheritance (Myers, 2004). The CAG repeat is unstable and is known to expand during spermatogenesis; the levels of CAG-repeat expansion in sperm cells has been reported to be extremely high (Myers, 2004). In some cases, an especially expanded CAG-repeat is inherited by the offspring (>50 CAG-repeats, with cases of 100 or more repeats in juvenile HD children attending the Queen Square HD Multi-disciplinary Clinic, Prof Sarah Tabrizi, personal communication). This leads to a subtype of HD called juvenile HD, which will be discussed in further detail.
1.1.3 Clinical presentation of HD

HD is typically characterised by a triad of symptoms that contribute to patient mortality, which usually occur within 15 years of initial disease diagnosis: personality and behavioural changes classically present first (Borgs and Godin, 2012; Julien et al., 2007); a progressive cognitive decline due to atrophy of the brain results in dementia (L.A. Raymond et al., 2011; Vonsattel et al., 2011); and finally, patients develop choreic movements which increase in severity in parallel to disease duration (Borgs and Godin, 2012). Alongside these defined symptoms, patients typically experience exhaustion, contributing to dramatic weight loss; the combination of all symptoms contributes to a gradual loss of independence over the course of the disease. Each patient is unique in their disease onset, symptom manifestation and progression, however CAG-repeat length is strongly inversely correlated with age of onset (Cowan and Raymond, 2006; Reddy and Shirendeb, 2012; Wilson et al., 2017) and disease severity (Pla et al., 2014). The typical natural progression of the disease and each of the disease stages is depicted graphically in Figure 1.1.

Figure 1.1. The typical natural progression of Huntington’s disease. A diagrammatic timeline of the sequence of events in HD disease progression. As toxicity and pathology accrue in the brain, functional ability declines. This occurs prior to the manifest stage the start of which is defined by the onset of motor impairment. Reproduced with permission from (Bates et al., 2015).

1.1.3.1 Motor symptoms

Initially HD was termed Huntington’s chorea, as chorea is one of the most prominent and recognisable motor features of the disease (Huntington, 1872). Chorea is the Greek term for dancing, which aptly describes this type of dyskinesia. Involuntary contractions of muscle groups lead to the execution of jerking and excessive movement commonly affecting the limbs
and the trunk. In some cases, these movements can appear directional, as though the individual is attempting to reach or grasp an object; HD patients can try to mask their choreic movements by attempting to disguise them with a semi-purposeful movement. There are arguments however for both a worsening, or improvement in choreic movements in correlation with increased voluntary actions (Fahn et al., 2007). Inability to sustain voluntary muscle contraction as well as excessive tongue protrusion are also some of the most commonly reported features of chorea (Fuller et al., 2010). These symptoms are typically observed in patients experiencing the hyperkinetic phase of HD, however as the disease progresses over time, there is a gradual reduction in the ability of HD patients to execute voluntary movements in general, leading to hypokinesia, bradykinesia, and rigidity (Ross and Tabrizi, 2011; Wichmann and Dostrovsky, 2011).

1.1.3.2 Cognitive symptoms
The cognitive symptoms that HD patients experience are often reported much earlier in the disease course, typically ahead of choreic movements. Indeed, it is usually cognitive deficiencies as well as psychological alterations that are first noticed by their relatives, and can be the most distressing and debilitating features of the disease (Craufurd and Snowden, 2002; Stout et al., 2011). Cognitive deficits include a reduction in executive functioning, and an impairment to both learning and memory. In later stages of the disease this can develop into clinical dementia (Mielcarek, 2015). Due to alterations in the limbic system and frontal lobe changes, response selection is affected in patient cohorts, typically manifesting in inappropriate emotive responses (Estrada-Sánchez and Rebec, 2013).

1.1.3.3 Psychiatric features
Finally, a host of psychological and mental health disorders are associated with HD. These include most commonly depression and anxiety, as well as a high prevalence of apathy (Craufurd and Snowden, 2002; Julien et al., 2007; Pla et al., 2014). There is debate as to whether the disease mutation is directly causative of these features, or if the HD diagnosis itself is attributable; the fact that the above features are also experienced by prodromal patients i.e. without a formal diagnosis, suggests the former, however as these patients have typically already tested positive for the HD mutation, there is perhaps an element of inevitability at play (Borgs and Godin, 2012; Julien et al., 2007). Although some of the psychiatric features above can be medically managed by selective serotonin reuptake inhibitors (SSRI) for example, they tend to worsen in correlation with disease progression and can contribute to mortality; suicide rates within the HD population are extensively elevated compared to the general population, with up to 30% experiencing suicidal ideals, up to 10% attempting to take their own life and up to 6.6% succeeding (Kachian et al., 2019). Personality features such as irritability, aggression and obsessive compulsive behaviour are also evident in the HD community, and are particularly distressing for HD families (Craufurd and Snowden, 2002).

1.1.3.4 Diagnosis
Diagnosing HD has been made simpler due to the availability of genetic tests. If a patient has a family history of the disease, they can elect to be genetically tested and have their risk status
revealed (Craufurd et al., 2015), termed ‘predictive testing’ (Losekoot et al., 2013). This is often quite a dilemma for individuals in an HD family, as although knowing their risk status is definitive in whether they will develop the disease, as yet there are still no known cures, therefore many individuals elect not to take the test. Despite the categorical nature of genetic testing, in which a polymerase chain reaction (PCR) is completed against the area encompassing the CAG-repeat, followed by fragment analysis and sizing based on international standards (Losekoot et al., 2013), a HD diagnosis and thus recording of age-at-onset (AAO) cannot be made unless the patient displays motor symptoms that are unequivocally attributable to HD and cannot be explained by any other cause. As such, HD patients may indeed experience cognitive and psychiatric symptoms decades before they are formally diagnosed with the disease (Bates et al., 2015; Zuccato and Cattaneo, 2014).

1.1.3.5 Disease management
As mentioned, to date there are no known disease modifying therapies for HD, which either modify the disease course and progression or cure the disease. Treatments are available to alleviate some of the symptoms of the disease, including antipsychotic medication and SSRI’s which combat some of the more debilitating psychiatric features (Pla et al., 2014; Ross and Tabrizi, 2011). In 2008, the monoamine depletory drug tetrabenazine was licensed to treat chorea in the United States and to date is the best known management for the motor symptoms experienced by patients (Huntington Study Group, 2006). As the disease course lengthens and disease severity increases, full-time care is required by the HD patient as daily tasks become too challenging to complete. As a result, a loss of independence is experienced which can contribute to the worsening of psychiatric features.

1.1.4 Juvenile HD
As aforementioned, support for positive correlation between CAG-repeat length and disease severity is presented by a second type, or subset, of HD termed juvenile HD, as symptoms present before the patient reaches 20 years of age (Bates et al., 2015). Juvenile HD is caused by much longer expansions of 50 CAG repeats and above (Cowan and Raymond, 2006). The disease is typically much faster in its progression (Cummings et al., 2009), and also displays contrasting motor symptoms to that of adult onset HD: bradykinesia, rigidity and seizures are prominent in juvenile HD patients (Vonsattel et al., 2011). Indeed, some of these symptoms mimic the motor phenotype presented by adult onset cases near the end-stage of disease, suggesting that juvenile HD is inherently more severe.

1.2 Huntingtin protein

1.2.1 Wild type protein function
Ubiquitously expressed throughout the body, wild type (WT) HTT has multiple diverse functions in normal physiology (Borgs and Godin, 2012). In 2018, a cryo-electron microscopy study unveiled the electron structure of WT HTT for the first time (Guo et al., 2018). The authors were successful in resolving the structure when HTT was bound to HTT-associated protein (HAP)40, and identified that the protein was largely composed of three domains: N- and C- terminal
domains which contained multiple HEAT (huntingtin, elongation factor 3, protein phosphatase 2A and lipid kinase TOR) repeats, which are connected by a bridge domain (Figure 1. 2) (Guo et al., 2018). This study confirmed the hypothesis that HTT is a highly interactive protein, as demonstrated by the resolving of many protein-protein interaction sites. Frequently described as a scaffold protein, HTT is intrinsically involved in axonal trafficking and microtubule associated cargo transport (Schulte and Littleton, 2011). HTT possesses multiple protein-protein interaction sites and has several interactor proteins, such as HAP1 and p150Glued, which form a complex that assists in vesicle trafficking along axons (Borgs and Godin, 2012; White et al., 2015).

Figure 1. 2. The molecular structure of HTT as resolved by cryo-electron microscopy (cryo-EM). In 2018, the structure of the WT HTT protein, when bound with HAP40, was resolved by cryo-EM. The structure revealed three main regions of the protein: two huntingtin, elongation factor 3, protein phosphatase 2A and lipid kinase TOR (HEAT) repeat rich domains, at the N- and C- terminus, which are connected by a bridge domain. Figure adapted from Guo et al., 2018.

One of the first roles discovered for HTT was its requirement for embryonic development: homozygote HTT knock-out mouse models are embryonically lethal by E8.5, and heterozygous HTT knock-out mice, whilst viable, demonstrate both physical and behavioural defects (Nasir et al., 1995). The fact that HTT plays a role in mitotic spindle formation could be one explanation for this embryonic lethality (Zuccato and Cattaneo, 2014). It is also found that WT HTT is required for neurogenesis (Godin et al., 2010a; Nguyen et al., 2013) and recent studies have identified that if knocked-out in human cells (embryonic stem cells), chromosomal instability occurs also resulting in impaired neurogenesis (Ruzo et al., 2018). In corticogenesis, Barnat et al., identified that HTT was required for newly born cortical progenitor polarisation and subsequent radial migration through the cortex, a process that was abolished upon the knock-down of HTT (Barnat et al., 2017).

Finally, the WT protein is believed to exert a neuroprotective role through a variety of mechanisms, including its inhibition of caspase-3 mediated apoptosis (Zhang et al., 2006), preventing cell death, as well as its facilitation of trophic factor production (see 1.6) and transport of said factors to networks of neurons within the central nervous system (CNS). The functions described above are limited to the CNS, however HTT is expressed in abundance throughout the peripheral nervous system and systemic tissues, and thus is known to have alternative roles throughout the body (reviewed in Mielcarek, 2015).
1.2.2 Mutant HTT protein gain/loss of function

In 1997 it was confirmed by Professor Bates' group that the mutant form of HTT is prone to aggregation and forms inclusions that are typically found in the cell nucleus (Davies et al., 1997). The contribution of mHTT inclusions to neurodegeneration is hotly debated. For example, it is hypothesised that these intranuclear inclusions may interfere with the cells ability to transcribe and translate essential proteins thereby contributing to cell death (Steffan et al., 2000). However, in some HD animal models, which will be discussed in more detail, symptoms manifest and persist in the absence of intranuclear inclusions (Raymond et al., 2011).

Secondary to the molecular alterations introduced by mHTT, the aggregates themselves can also hinder cellular functionality in a physical manner. The mHTT aggregates formed in both the nucleus and cytoplasm can have drastic blockage effects within the cell. There have been studies suggesting that the accumulation of aggregates within axon end points and synaptic terminals induces synaptic degeneration; action potential propagation is hindered resulting in reduced communication between neurons, and subsequent synaptic degeneration (Vonsattel et al., 2011). Secondary to the loss of synaptic transmission, the degenerating synapse sends out ‘distress signals’ such as danger-associated molecular patterns (DAMPs) to the brain’s resident immune cells, microglia, as well as astrocytes (Puig et al., 2018), which act in tandem to clear the debris left and create a pro-inflammatory environment in the surrounding area. This can lead to further damage to surrounding neurons if not resolved quickly (Frank-Cannon et al., 2009).

In HD, mHTT has been shown to impair several aspects of axonal trafficking in multiple models, including Drosophila, HD mouse models and cultured neurons (Gunawardena et al., 2003; Smith et al., 2014; White et al., 2015). A second structural deficit that is induced by the presence of aggregated HTT, is the blockade of transport of vital organelles such as mitochondria throughout the cell and along axons to synaptic terminals (Davies et al., 1997; Han et al., 2010). The presence of mitochondria in synaptic terminals is extremely important, as not only do they aid with synaptic transmission and ionic homeostasis, but also facilitate active transport across the membrane to re-uptake neurotransmitters and restore the resting membrane potential. As such, when mitochondrial trafficking is deficient, synaptic degeneration and death can also occur due to energy failure, resulting in a similar pro-inflammatory state as described above (Borgs and Godin, 2012).

One aspect of HD pathology that is still not well understood is that of neuronal vulnerability, and why some brain regions appear to succumb to the toxic effects of the protein more rapidly and in greater severity than others (Cowan and Raymond, 2006).

1.3 Huntington’s disease pathology

1.3.1 General overview

The pathophysiological hallmarks of HD are well defined: mHTT aggregates can be detected throughout the brain and post-mortem samples show decreased brain weight as a result of globalised atrophy in both grey matter and white matter. These correlate with a decline in cognitive and motor performance in patients (Hobbs et al., 2010). The primary areas of cell loss
However are the striatum and regions of the cerebral cortex which will be discussed in further detail (Bates et al., 2015; Novak and Tabrizi, 2010; Ross and Tabrizi, 2011). Imaging studies have been integral to the discovery of altered brain physiology in HD patients (Wilson et al., 2017), with some changes noted prior to symptom manifestation. Indeed, changes in white matter integrity occur even at pre-manifest stages and correlate with disease progression (Novak et al., 2014). Furthermore expression of phosphodiesterase 10A (PDE10A), which is located primarily in striatal neurons, is documented by positron emission tomography (PET) studies to be decreased in excess of 25 years prior to symptom onset, indicating that the toxic effects of mHTT within the brain are accumulating decades before symptom manifestation and clinical diagnosis (Niccolini et al., 2017, 2015).

1.3.2 Globalised atrophy and pathology

The average weight of a human brain at post-mortem, with no clinical pathologies or disease diagnosis, is approximately 1350 g (Vonsattel et al., 2011); this value is influenced of course by age and sex differences. In contrast, the brain of a HD patient upon post-mortem is on average 300 g less than this value (de la Monte et al., 1988; Vonsattel et al., 2011), indicating the large scale global atrophy of the brain that occurs during the disease. By assessing post-mortem samples in further detail, specific brain regions have been identified as being particularly vulnerable to atrophy as previously mentioned, however the loss of both grey and white matter throughout the cortices, deep brain regions and brain stem should not be overlooked. Indeed, in a large quantitative anatomical study of moderate HD cases, a 21-29% reduction was observed in cerebral cortex, 28% in thalamus, 57% in the caudate, and 64% in the putamen. Recently however, atrophy of the cerebellum has been identified in the disease, and the degree to which the cerebellum atrophies is in correlation with the severity of motor symptoms; the cerebellum is a structure that was previously thought to be spared in the disease (Rüb et al., 2013; Singh-Bains et al., 2019).

1.3.2.1 Vonsatel grading

In 1985, Jean Paul Vonsatel pioneered a grading system that could be applied to HD brains to indicate the severity of the disease at post-mortem. The Vonsatel system is still used in research to date, and uses a combination of macroscopic and microscopic parameters to classify a wide variety of brain regions, including the caudate nucleus, cerebral cortex and brain stem (Vonsattel et al., 1985). The macroscopic analysis involved measuring the size, shape and volume of the caudate nucleus and putamen to determine the levels of atrophy. Microscopic features included calculating the ratio of neuronal density and astrocytic population, as well as determining the proportion of astrocytes within a region that were considered reactive. The scale for these measures formulated a grading system from 0 to 4, as described in Table 2.

Table 2. Grading system developed by J P Vonsatel detailing macroscopic and microscopic features of HD brains.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Macroscopic features</th>
<th>Microscopic features</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No alteration to striatal volume or size</td>
<td>Normal neuronal density and astrocytosis within normal</td>
</tr>
<tr>
<td></td>
<td>Striatal Pathology</td>
<td>Marked Brain Pathology</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>No alteration to striatal volume or size</td>
<td>Slightly elevated astrocytosis, neuronal density within normal limits</td>
</tr>
<tr>
<td>2</td>
<td>Slightly atrophic putamen, variable atrophy of caudate nucleus</td>
<td>Marked neuronal loss and elevated fibrillar astrocytosis</td>
</tr>
<tr>
<td>3</td>
<td>Lateral ventricle enlargement, reduced size and altered shape of the caudate nucleus, putamen and globus pallidus</td>
<td>Marked neuronal loss and high levels of astrocytosis in caudate nucleus, putamen and globus pallidus</td>
</tr>
<tr>
<td>4</td>
<td>Shrunken caudate nuclei, atrophic putamen and globi pallidi, striking enlargement of lateral ventricles</td>
<td>Severe neuronal loss throughout the entirety of the striatum, evident astrocytosis, altered structure and organisation of the globus pallidus</td>
</tr>
</tbody>
</table>

### 1.3.2.2 Reactive gliosis

As documented by Vonsatel, a high level of astrocytosis is observed in the HD brain, increasing in severity in concordance with disease progression. In tandem with microglia, the brains resident immune cells, an immune response can be activated causing astrocytes to turn reactive, a process termed reactive astrogliosis. Both the WT and mHTT protein are expressed by astrocytes, and previous reports have identified that these cells have altered functionality as a result of the toxic protein, including reduced expression of the glutamate transporterGLT1 at both the mRNA and protein level (Wood et al., 2019), and reduced ability to release brain derived neurotrophic factor (BDNF) (Hong et al., 2016). Microglia, which provide the main inflammatory response in the CNS to an infection or insult, are also affected by their expression of mHTT. An elevated number of activated microglia have been identified both in HD patient brains and mouse models of the disease (Franciosi et al., 2012; Sapp et al., 2001; Yang et al., 2017). Furthermore, elevated levels of inflammatory cytokines released by microglia have been found in both cerebrospinal fluid (CSF) and plasma of HD patients, in some cases before symptom manifestation and formal diagnosis, indicating that there is an elevated level of neuroinflammation early in the disease (Politis et al., 2015; Yang et al., 2017). Although much less common than in neurons, mHTT aggregates have been found in both microglia and astrocytes in HD animal models, thereby suggesting that structural toxicity could be accumulating in these cells also (Jansen et al., 2017).

### 1.3.3 Specific regions of brain pathology

As evident from the Vonsatel grading system described above, the area of the brain that undergoes the most atrophy during disease progression is the striatum. Indeed, this is the brain
area in which atrophy can be first observed, and as demonstrated by grade 4 HD cases, up to 95% of the structure in its entirety is lost. The striatum is comprised of medium spiny neurons (MSNs), which make up 95% of the structure, with the remaining 5% comprising aspiny interneurons. Therefore, as we see a 95% reduction of striatal volume and integrity at end-stage cases, we can assume that nearly all MSNs are lost in this disease (Vonsattel et al., 1985, 2011). A dramatic enlargement of the lateral ventricles is also evident, accompanied by the loss of volume in surrounding structures. In an extensive study conducted by Rosas et al., it was found that the ventricles were enlarged by over 100% in an HD cohort compared to control subjects (Rosas et al., 2003).

Throughout the cerebral cortex, a reduction of grey matter thickness and integrity can be observed by histochemistry and imaging techniques, which is also compounded by a reduced thickness of corresponding white matter. Specific layers of the cortex, however, appear to be more vulnerable than others due to the toxicity accrued in the disease. Cortical layer V, which contains cortical projection neurons (CPN) has particularly striking evidence of atrophy, as well as layers III and VI to a lesser extent (Reiner et al., 2011). Depending on the location within the brain i.e. deep or superficial, white matter loss occurs at a different rate, not only in terms of speed of degeneration, but also in the disease time course (Wu et al., 2017). An interesting study published by Douaud et al., postulated that the selective degeneration of cortical layers and subcortical regions originates from the degeneration of the striatum; due to the extensive connectivity within cortico-striatal-thalamo-cortical loops, the cortical areas affiliated were also susceptible to degeneration. This could be one possible explanation for the overt degeneration observed in cortical layer V neurons, as these are the principle neurons that project to the striatum and form the corticostriatal (CS) pathway (Douaud et al., 2009). Taken together, the spread of pathology from the striatum to deep white matter tracts and cortical regions implies that seeding may be taking place. The concept of seeding is mostly associated with prion disease; fragments of toxic protein are released from diseased cells and ‘infect’ cells in their surrounding environment by deposition. These fragments then act as ‘seeds’, propagating disease pathology (Tan et al., 2015). The idea that mHTT pathology can spread in a prion-like fashion in the brain is becoming increasingly investigated, and would explain the sequence of spatial pathology spreading within the HD brain (Pecho-Vrieseling et al., 2014; Zhang et al., 2011).

1.3.4 Corticostriatal pathway

The CS pathway, therefore, is particularly sensitive to the effects of mHTT and succumbs to toxicity-induced degeneration relatively early in the disease process. The CS pathway is comprised of layer V CPNs and MSNs of the striatum. Cortical neurons project their axons through the brain parenchyma to synapse with their targets, the dendritic spines of MSNs, thus providing them with excitatory innervation. There is strong evidence for disturbed CS connectivity in HD, with data from both rodent models (Bates et al., 2015; McKinstry et al., 2014) and human patients (McColgan et al., 2015) suggesting this breakdown in connectivity is at least partially responsible for the movement disorders observed in patients. The principle
function of the CS pathway is to modulate the activity of the basal ganglia circuitry and regulate its stimulatory or inhibitory drive to the thalamus. This in turn regulates thalamic-driven stimulation of the motor cortex, and enables the generation of fine motor control and movement (Figure 1.6). Thus, when CS connectivity is perturbed, precise control of the motor cortex is lost and disruption to normal movement is evident. The two main cell types that comprise this pathway will now be discussed in further detail.

1.4 Striatal medium spiny neurons

MSNs are inhibitory cells (gamma-aminobutyric acid [GABA]ergic) that comprise 90-95% of the neuronal population of the striatum (Haber, 2016; Morigaki and Goto, 2017); the remaining 5-10% is made up of interneurons of which there are four classes (Cepeda et al., 2007). Developmentally, MSNs arise from the neuroectoderm, specifically from cells destined to produce the ventral telencephalon, sometimes known as the subpallium (Campbell, 2003; Carri et al., 2013). The subpallium is committed to form three structures, one of which is the lateral ganglionic eminence and it is from this developmental structure that MSN precursors arise. Once terminally differentiated, striatal neurons can be identified by the specific combination of genes and proteins they express. High expression of dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32) - a key mediator of dopamine signalling (Precious et al., 2016; The HD iPSC Consortium, 2012) - combined with COUP TF1-interacting protein 2 (CTIP2) - a transcription factor essential for MSN differentiation (Arlotta et al., 2008) - typically identifies MSNs. Furthermore, the unique electrical properties attributed to MSNs allows for their identification in vitro. The cells have a low spontaneous firing rate but are capable of fast repetitive firing with stimulation. A well defined characteristic of MSNs is the slow depolarization state and thus delayed action potential firing of initial spikes (Figure 1.3), which is caused by the activity of fast-inactivating potassium (K+) channels which replicate I_A currents (Carri et al., 2013; Ericsson et al., 2011). MSNs are the principle output nuclei of the basal ganglia and thus create complex and dense networks both with neighbouring MSNs, as well as their external targets (Chuhma et al., 2011).

Figure 1.3. Example image of typical MSN found in culture and schematic of delayed latency to fire. Left: an iPSC-derived MSN transfected with GFP and aged to 50 days in culture. The picture shows advanced dendrification as well as numerous dendritic spines. Right: an example of a typical action potential trace expected when recording from mature MSNs.
Within the basal ganglia there are multiple feedback loops that regulate the basal ganglia activity internally and consolidate output signals. MSNs can be segregated into two distinct pathways dependent on their synaptic targets, termed the direct and indirect pathway (Gerfen, 1988; Silberberg and Bolam, 2015). The subtypes are distinguishable by the combination of genes and proteins they express, basal activity levels (Gertler et al., 2008) and at least in part by their morphology (Tinterri, 2016). The activity of the two pathways in unison enables individuals to produce controlled, coordinated movements through the activation of specific muscle groups and the inhibition of others.

### 1.4.1 Direct pathway MSNs

The direct pathway refers to the innervation loops originating from MSNs that express dopamine receptor 1 (DRD1) and substance P (SubP). Direct pathway MSNs facilitate movement via downstream basal ganglia signalling and regulation of thalamic activity. Anatomically, direct pathway MSNs are highly complex neurons with densely arborised dendritic trees, each with a large number of dendritic spines, hence the name medium spiny neurons (Figure 1. 3.). As aforementioned, direct pathway MSNs express DRD1 as its predominant dopamine receptor alongside muscarinic M4 receptors. The neurons also express the neuropeptides dynorphin and substance P, which taken together allow for their identification (Kreitzer, 2009).

### 1.4.2 Indirect pathway MSNs

The indirect pathway MSNs, although also GABAergic and thus inhibitory, have a different expression profile, which enables researchers to distinguish between cell types of the two pathways. These MSNs express dopamine receptor 2 (DRD2) as well as adenosine A2A receptors (A2AR) on their cell membranes and contain the neuropeptide enkephalin. Indirect pathway MSNs are morphologically indistinguishable from direct pathway MSNs and display very similar membrane properties, thus immunoreactivity and gene expression analysis is the only way in which the two populations can be identified in culture or in vivo thus far (Gerfen, 1988; Kreitzer, 2009).

### 1.4.3 Striatal compartmentalisation and cellular location

The striatum itself can be functionally divided into dorsal and ventral segments; MSNs have been positively identified in both regions. Whilst a random distribution of direct and indirect pathway MSNs is found in the dorsal striatum (Gangarossa et al., 2013b), the ventral striatum, also known as the nucleus accumbens, displays a more complex and regimented structure (Figure 1. 4). The ventral striatum itself can be further divided into ‘core’ and ‘shell’ regions and whilst direct pathway MSNs appear evenly distributed across the entirety of the ventral striatum, DRD2 expressing MSNs i.e. indirect pathway, are found in lower density in the ventral shell, whilst the medial region of the shell was found to contain high number of DRD2 expressing MSNs (Gangarossa et al., 2013a); within the core, an even distribution of DRD2 expressing MSNs was observed (Gangarossa et al., 2013a; Tinterri, 2016).
MSNs can be identified in the compartments however previous studies in mice have identified subtle differences in expression levels of SubP and enkephalin, suggesting that the ratios of direct, and indirect-pathway MSNs within the distinct compartments may be slightly different. A higher expression of SubP has been found in the striosome compartment, suggesting elevated levels of direct pathway MSNs, conversely enkephalin appears enriched in matrix compartments suggesting a higher population of indirect pathway MSNs (Figure 1. 4). Interestingly, there is debate as to whether the striosome and matrix compartments receive the same innervation from the cerebral cortex, with studies suggesting that limbic structures preferentially target striosomes whilst motor cortices target the matrix (Fujiyama et al., 2015).

**Figure 1. 4. Simplified diagram of the human striatum.** The striatum can be segregated into two main compartments: the striosomes, depicted by pink shapes, and the matrix shaded in light blue. Indirect pathway MSNs (black circles) are found in higher abundance in striosomes, whilst the matrix contains more direct pathway MSNs (white circles). The ventral striatum, or nucleus accumbens, is composed of ‘core’ and ‘shell’ compartments. Direct pathway neurons (white) are spread evenly across both core and shell but indirect pathway neurons (black) are found less frequently in the shell.

### 1.5 Cortical projection neurons

#### 1.5.1 Layers of the cortex

The cerebral cortex is a complex structure that can be delineated into different functional areas such as the frontal cortex or motor cortex, and also into six distinct cellular layers depending on the neuronal subtype that resides there (Kandel et al., 2000; Molyneaux et al., 2007). The neurons within each cortical layer differ in their morphology, receptor expression and activity, which is reflected in their differing functions within the brain (Estrada-Sánchez and Rebec, 2013). During development the layers of the cerebral cortex are generated in a time-dependent manner, with deep-brain layers V and VI emerging first (Molyneaux et al., 2007). Complex intercellular signalling mechanisms initiate a switch in developmental fate specification and
allow for the more superficial layers IV-I to emerge at later developmental time points (Molyneaux et al., 2007; Muralidharan et al., 2017; O'Leary and Nakagawa, 2002).

1.5.2 Layer V projection neurons
The cortical neurons that comprise part of the CS pathway have been identified as layer V projection neurons that are glutamatergic and therefore excitatory. These neurons can be further divided into two classes dependent on their morphology and their differential targets within the striatum: pyramidal tract (PT)-type neurons originating from lower layer V project primarily to indirect pathway MSNs, whilst intra-telencephalic (IT)-type projection neurons innervate direct pathway MSNs (Estrada-Sánchez and Rebec, 2013). As well as providing glutamatergic innervation, cortical neurons also provide trophic support to MSNs, which is essential for their survival and continued functionality. BDNF is produced by cortical neurons and trafficked anterogradely along axons to striatal terminals. It is then secreted in response to neuron depolarisation and once bound post-synaptically, initiates tropomyosine kinase receptor B (TrkB)-mediated signalling in striatal neurons (Baydyuk and Xu, 2014; Zuccato and Cattaneo, 2007). As previously mentioned, cortical axonal transport has been shown to be disrupted in HD animal and models. Furthermore, there is also evidence to suggest that mHTT interferes with the expression of both the BDNF transcript and protein in cortical neurons. For this reason, BDNF biology is a highly investigated area of research in the HD field. However, the examination of BDNF within human cortical neurons in the context of HD and CS connectivity has so far not been reported.

1.6 Brain derived neurotrophic factor

1.6.1 Protein production and processing
In humans, the BDNF gene has been mapped to chromosome 11 and contains ten exons, only the 3’ of which encodes the BDNF protein sequence (Binder and Scharfman, 2004). A total of eight unique transcripts can be produced. BDNF is produced as a proneurotrophin, which is cleaved by multiple enzymes, including prohormone convertase 1 (PC1), to its mature form after shuttling from the trans Golgi network intracellularly, and matrix metalloproteases that cleave the pro-domain extracellularly once secreted (Borodinova and Salozhin, 2017; Yang et al., 2009). From primary culture models, it is known that an increase in intracellular calcium as a result of neuronal activity increases the transcription of BDNF, thus it is commonly thought of as an activity-dependent protein (Hartmann et al., 2001; Zuccato and Cattaneo, 2007). Furthermore, its secretion and release is also partially regulated by activity, as exocytosis of the protein from dense core vesicles (DCV) occurs at axon terminals upon action potential propagation, although there is evidence for a small level of constitutive release (Kuczewski et al., 2009).

1.6.2 Protein function
Both the mature and pro- forms of BDNF have biological function, which appear to oppose each other in many cases (Li et al., 2017). The reason for this is that each protein binds with different affinity to one of two receptors, TrkB, or the tumour necrosis factor receptor, p75. Activation of
each of these receptors initiates diverse intracellular signalling cascades, resulting in different biological adaptations and functions (Figure 1. 5). The mature BDNF protein binds with high affinity to TrkB which is a transmembrane receptor. Upon BDNF binding, the BDNF-TrkB complex forms a heterodimer and autophosphorylation occurs resulting in recruitment of intracellular adaptor proteins. At this point, three intracellular signalling cascades can be activated including: the MAPK-ERK pathway, which acts to regulate gene transcription at the single cell level; the PI3-AKT1 pathway, which promotes cellular survival neurite growth and proliferation; and activation of phospholipase C (PLC) which regulates synaptic plasticity (Barnabé-Heider and Miller, 2003; Borodinova and Salozhin, 2017; Reimers et al., 2014).

In contrast, whilst proBDNF binding to p75 also leads to activation of intracellular signalling cascades, namely the NF-κB-dependent and JNK-dependent pathways, these lead to programmed cell death, and the Rho-RhoA pathway which is intrinsically involved with the actin cytoskeleton, and can lead to structural disruption (Borodinova and Salozhin, 2017; Li et al., 2017; Qiao et al., 2017; Yang et al., 2014, 2011). It is unsurprising therefore that evidence is mounting to suggest that mature BDNF and pro-BDNF have opposing roles in other biological processes, such as cellular migration, neurite growth and dendritic branching, whereby BDNF enhances and stimulates these processes, and pro-BDNF hinders them (Borodinova and Salozhin, 2017; Li et al., 2017; Qiao et al., 2017; Yang et al., 2014).
Figure 1.5. BDNF-TrkB and proBDNF-p75 downstream signalling pathways. BDNF binds with high affinity to TrkB receptors located on post-synaptic membranes. Here, three intracellular signalling cascades are initiated, including the MAPK-ERK pathway, the PI3-Akt pathway and activation of phospholipase C (PLC). ProBDNF binds with higher affinity to p75 receptors, which initiates NF-kB, JNK and RhoA signalling pathways. Figure based and adapted from (Begni et al., 2017).

1.6.2.1 Cortical neuron BDNF production

It is well established that cortical neurons, and cortical progenitor cells synthesise and secrete BDNF, which can function in not only a paracrine, but also autocrine way. Indeed it was observed by Barnabé-Heider and Miller (2003) that when the downstream signalling pathways of TrkB were inhibited in isolated progenitor cultures, cortical progenitor cell death was elevated, indicating that endogenous BDNF is important for progenitor cell survival and neurogenesis (Barnabé-Heider and Miller, 2003). Cortical BDNF production is also important in the context of the CS pathway, as the protein is trafficked anterogradely along cortical axons to synaptic terminals in proximity of the striatum. Previous publications have co-localised BDNF to proteins such as secretogranin II (SG-II) found in DCVs (Wu et al., 2004), therefore it is concluded that these vesicles are the principle mechanism by which BDNF is shuttled throughout the cell (Borodinova and Salozhin, 2017). It is also interesting to note that in order to be sorted effectively from the endoplasmic reticulum - the site of protein synthesis - proBDNF forms a complex with huntingtin-interactor protein 1 (HAP1), with evidence suggesting that particularly in cortical neurons, if formation of this complex is inhibited, levels of BDNF are reduced within the
cell (Yang et al., 2011), which has important implications for cortical BDNF production in HD as discussed later.

1.6.2.2 MSN BDNF requirements

As aforementioned, the binding of mature BDNF to the TrkB receptor initiates several intracellular signalling cascades which act to regulate cell survival and plasticity. A key feature of striatal neuron biology is that they are unable to produce mature BDNF themselves, therefore depend on exogenous uptake (Apostol et al., 2008; Zuccato and Cattaneo, 2007). Cortically produced BDNF delivered via the CS pathway is the principle source of BDNF for striatal neurons post-natally (Baquet et al., 2004). One of the key functions of BDNF in striatal neurons is to regulate and direct synaptic plasticity; BDNF-mediated signals alter the neurotransmitter receptor expression levels at the cell surface including for example AMPA receptors (Reimers et al., 2014), thereby contributing to the process of learning and memory. The neurotrophin is also integral to neuritogenesis and subsequent pathfinding ability of striatal neurons, as such it also plays a role in establishing striatal network complexity (Baydyuk and Xu, 2014). Finally, due to BDNF-mediated actin cytoskeleton remodelling, BDNF signalling can drive an alteration in the density and morphology of dendritic spines present on striatal processes, thereby altering the excitability and sensitivity of the cells as a whole (Orefice et al., 2013).

1.6.3 BDNF production and trafficking

Wild type HTT plays a role in upregulating BDNF transcription (Zuccato et al., 2001) via its transcriptional repression of the neuron restrictive silencer element (NRSE) (Zuccato et al., 2003), and the presence of an expanded HTT polyQ is detrimental to BDNF transcription. Therefore it is unsurprising that in both animal models of the disease (Zuccato et al., 2005), as well as human post-mortem brain tissues, reduced levels of BDNF are observed (Zuccato et al., 2007). Furthermore, live imaging studies have provided clear evidence of reduced BDNF trafficking in both the anterograde and retrograde direction (Baydyuk and Xu, 2014; Nithianantharajah and Hannan, 2013; Raymond et al., 2011). These deficits are compounded by a reduced vesicle release potential identified in primary cortical neurons isolated from the HdhQ140 mice suggesting that overall BDNF processing is defective in HD (Virlogeux et al., 2018).

1.6.4 Striatal BDNF uptake, trafficking and signalling

Whilst there are abundant publications noting dysfunctional BDNF processing on the cortical side of the CS pathway, it should not be overlooked that MSN uptake and post-synaptic signalling is also abnormal in HD. An elegant study conducted by Giné et al., in 2006 identified that in three genetic HD mouse models a reduced TrkB protein expression was found in the striatum. This was corroborated by a reduced expression of TrkB in an immortalised HD striatal cell line (Giné et al., 2006). Aside from the expression of the receptor itself, evidence suggests that the functionality, trafficking and signalling of BDNF post-synaptically is also abnormal. Liot et al., found that in the healthy mouse brain, once TrkB bound BDNF and was internalised within striatal dendrites, it formed a complex with HTT itself as well as motor protein dynein, facilitating its retrograde trafficking along microtubules. However, in a disease context where an
expanded polyQ-tract was present, this complex formation, was inhibited and thus destabilised the cargo from microtubules and disrupted retrograde trafficking (Liot et al., 2013). As a result, the induction of downstream BDNF signalling pathways - ERK phosphorylation and induction of c-fos - were reduced by nearly 50%, suggesting that intracellular signalling is significantly compromised in MSN dendrites. The consequences of these deficiencies are yet to be evaluated fully, however a reduction in BDNF availability via the CS pathway, compounded with reduced striatal uptake and signalling is why BDNF is one of the main foci in the HD research field, and has been put forward as a primary mechanism to explain the selective vulnerability of MSNs in HD (Baydyuk and Xu, 2014).

1.7 Corticostriatal connectivity

1.7.1 The CS pathway in the healthy brain

In the healthy brain, the CS pathway acts in a highly controlled manner to regulate basal ganglia signalling and downstream effects. The innervation loops discussed are depicted graphically in Figure 1. 6. Briefly, in the context of regulating the motor cortex: once an excitatory signal is received in the cortex, this is propagated along layer V CPNs to the striatum onto both direct and indirect pathway MSNs. Firstly, addressing the direct pathway, MSNs increase inhibition of the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr). The cells in these regions usually provide tonic inhibition to the thalamus therefore when they themselves are inhibited, there is reduced thalamic inhibition resulting in stimulation of the motor cortex and movement (Dautan, 2016). The direct pathway is hence described as a ‘GO’ signal, as in the typical healthy individual, it stimulates movement (Figure 1. 6.A).

Conversely, the indirect pathway can be thought of as the ‘STOP’ signal, as signalling through this pathway results in the termination of movement (Figure 1. 6.B). Indirect pathway MSNs, once stimulated by CS afferents, project to the external GP (GPe) and exert an inhibitory input thus rendering the GPe inactive. Usually inhibited by the GPe, the excitatory neurons of the subthalamic nucleus (STN) are thus free to stimulate the cells of the GPi and SNr resulting in increased inhibitory drive to the thalamus and reduced stimulation of the motor cortex, which acts to restrict movement (Chuhma et al., 2011).
As aforementioned, the remaining 5-10% of striatal neurons can be identified as either GABAergic or cholinergic interneurons. These function to facilitate signalling between direct and indirect pathway MSNs, hence further refining control of thalamic and motor cortex activity (Ramanathan et al., 2002). There is also evidence to suggest that a subset of cortical projection neurons synapse directly with these interneurons and not with MSNs, which is hypothesized to facilitate the convergence and integration of information from different areas of the cortex prior to passing that information on to the striatum (Haber, 2016). Interestingly, striatal interneurons do not appear to degenerate in a similar manner to MSNs, despite also expressing mHTT, a phenomenon that is currently under investigation (Han et al., 2010; Morigaki and Goto, 2017).

There is another pathway involved in basal ganglia signalling termed the hyper-direct pathway, as this does not involve MSN activity at all, and is in fact a direct connection of frontal cortical neurons to the STN (Haber, 2016). Finally, dopaminergic control of direct and indirect pathway MSNs is exerted by the substantia nigra pars compacta (SNc). Signalling through this structure ultimately acts to stimulate movement, as dopamine acts in an inhibitory manner on the indirect pathway, but in an excitatory manner on the direct pathway (Rangel-Barajas and Rebec, 2016).
1.7.2 The CS pathway in the HD brain

In the context of HD, these pathways are disrupted and substantial evidence shows that the MSNs of the indirect pathway are more vulnerable to the effects of mHTT than direct pathway MSNs (Baydyuk and Xu, 2014; Cowan and Raymond, 2006; Ehrlich, 2012; Raymond et al., 2011), which succumb to toxicity later on in disease progression. Higher endogenous HTT expression within indirect MSNs could explain this difference (Fusco et al., 2003). The sequential degeneration of indirect pathway followed by direct pathway MSNs is reflected in the evolution of movement disorder symptoms, which progress from hyperactivity to bradykinesia and rigidity in concordance with typical disease progression and severity.

There is still debate as to where the initial disruption occurs within the pathway and what the principle driver of pathology is (Morigaki and Goto, 2017). Although it is not currently clear whether the root cause for pathology is a breakdown in excitatory innervation, the inability to process and propagate signals, or degeneration of the MSN itself, still the changes to downstream basal ganglia signalling are well known. In the context of the motor phenotype, for example, a reduced output from indirect pathway MSNs enables GPe neurons to become active. These neurons then inhibit the activity of STN cells resulting in a lack of stimulation of the neurons comprising the GPi/SNr. This results in the loss of tonic inhibitory drive to the thalamus, resulting in thalamic over-activity and further downstream effects i.e. excessive and involuntary movement (Figure 1.7A).

In later stages of the disease, the direct pathway MSNs also degenerate which results in constant tonic inhibition of the thalamus, due to a lack of inhibition of the GPi/SNr. As such, the thalamus is inhibited and reduced downstream activation of brain regions such as the motor cortex is observed (Figure 1.7B). This is why the typical progression of movement disorder symptoms in patients evolves from hyperkinesia as a result of indirect pathway neuron degeneration, through to bradykinesia and eventually rigidity when the direct pathway neurons malfunction.
1.7.3 Contribution to motor phenotype

In HD, the contribution of the CS pathway and its breakdown in connectivity to the motor phenotype presented by patients is well understood. Degeneration of the indirect pathway, leading to a reduced inhibitory drive to the thalamus in early stages of the disease, causes overactivation and stimulation of downstream thalamic targets such as the motor cortex. Thus, when overstimulated we observe the manifestation of uncoordinated, involuntary movements lacking fine control, that are classically experienced by HD patients. Furthermore, as the disease progresses and the DRD1 positive population also diminishes in patient brains, we begin to see a gradual reduction in all movement, leading to bradykinesia, rigidity and inability to commence movement. In end-stage patients, dysphagia is common due to a loss of muscle tone in the pharyngeal muscles, as such pulmonary and bronchial infection is frequent; pneumonia is in fact one of the leading causes of death for HD gene carriers, rather than the disease itself (Heemskerk and Roos, 2012).

1.7.4 Contribution to cognitive phenotype

The CS pathway is also involved in executing higher cognitive functioning, therefore it is hypothesised that the breakdown of CS connectivity contributes to the cognitive changes experienced by patients (Craufurd and Snowden, 2002). Whilst the dorsal striatum is principally important for motor cortex information processing, cortical projections from limbic structures...
preferentially target the ventral striatum (Petrasch-Parwez et al., 2012), primarily the striosome compartment (Mehrabi et al., 2016). The fronto-striatal loops present in the brain appear to be compromised in HD and are hypothesised to be responsible for diminished executive functioning, psychomotor functioning, perceptual and spatial skills and memory (reviewed in Craufurd and Snowden, 2002; Julien et al., 2007).

1.8 Modelling HD
Due to the inaccessibility of human neural tissue samples from living HD gene carriers, animal models are necessary in order to study HD pathology on a cellular and molecular level. There are a wide variety of model species to choose from including pigs (Yan et al., 2018; Yang et al., 2010), sheep (Jacobsen et al., 2010), rats (Carreira et al., 2013) and the overwhelming popularity of using HD mouse models, of which there are many and will be discussed further below. Regarding in vitro studies, cell models of the disease have also been developed, which are particularly useful for studying neural-specific aspects of the disease.

1.8.1 Animal models
As mentioned, the majority of HD research in animals to date has been conducted using mouse models of the disease (Ferrante, 2009), however there are many different types of genetic HD mouse model that can be classified as either transgenic, encompassing both fragment and full-length models, as well as knock-in models. Each have their advantages, but no HD mouse model is perfect and instead should be selected carefully in order to best address the scientific question at hand.

1.8.1.1 Mouse models of HD
The most widely used mouse model to date in the HD field is undoubtedly the R6/2 exon 1 model, in which the human exon 1 fragment containing around 150 CAG-repeats is expressed alongside both copies of the murine huntingtin gene Hdh. As the name suggests, this is a fragment model of HD and has an extremely aggressive phenotype, with manifestation of symptoms as early as four weeks of age, and mortality typically between 14-16 weeks of age (Mangiarini et al., 1996). Due to the early onset and rapid disease progression in this model, it is frequently selected for research. However, due to the very large CAG-repeat length incorporated into the mouse genome, which frequently undergoes expansion (to repeat-lengths in excess of 250), there is debate as to whether this model is actually more representative of juvenile HD rather than the more typical adult onset (Ferrante, 2009). In contrast, full-length transgenic mouse models of HD, including the yeast artificial chromosome (YAC) (Hodgson et al., 1999), and bacterial artificial chromosome (BAC) models (Gray et al., 2008) have a much more representative disease progression. Subtle phenotypes first manifest around two months of age, and progress over the course of twelve months. Finally, knock-in models have been developed in which the human HTT sequence has been inserted into the native murine homologue Hdh, thus expression of human HTT is under the control of the murine promoter (Shelbourne et al., 1999).
All of the above models recapitulate some of the phenotypes and symptoms of the human disease, including brain atrophy, weight loss, motor and cognitive deficits and premature death. However, each of the above models also have their own caveats and phenomena that are not typically described in the human disease: as the most aggressive model, R6/2 mice have much wider spread pathology (Cummings et al., 2009) than exhibited in human HD brains, with mHTT aggregates observed in nearly all brain regions; YAC mice expressing full-length HTT containing 128 CAG-repeats exhibit weight gain (Van Raamsdonk et al., 2006); BACHD mice appear to manifest symptoms in the absence of abundant aggregate load, which again begs the question of the toxicity of the actual aggregates themselves. As knock-in mouse models express the human mHTT transcript endogenously, typically the phenotypes observed are much less severe, and the disease course can be much extended. As such, these mice are challenging to use for therapeutic studies as there is less ‘room for improvement’, so to speak, as the phenotypes are not extreme even in diseased mice. One caveat that cannot be overlooked when it comes to animal models of HD, is just that, they are animal models of a human disease, and of course do not develop HD naturally, thus cannot be expected to fully recapitulate a disease that only occurs in humans.

1.8.2 Cell models

A whole range of immortalised cell lines, mostly of transgenic origin, have been used to study HD to date. Rodent primary cells are popular tools for use, as is the immortalised mouse striatal STHdh line, which has been heavily used in the literature (Trettel et al., 2000) Another cell model used in the field of HD is that of a human immortalised neural progenitor line produced by ReNeuron, termed ReNCell (Donato et al., 2007; Pollock et al., 2006). This cell line readily differentiates into neurons and glia in culture, thus can quickly and efficiently produce the material required to study phenotypes on a cellular level. The cell line is amenable to transient and stable transfection, thus can be manipulated to express any number of CAG-repeats (within experimental and technical limits) thus these cells have previously been used to study HD pathology. With that said, Ghosh et al (submitted 2019) struggled to find mHTT-dependent cytotoxic phenotypes in a cellular cohort with either 71, or 129 CAG-repeats aside from mHTT aggregation and mitochondrial dysfunction, suggesting that ReNCells may not be the best model in which to study disease.

Over a decade ago, it became possible to generate human cell lines of any germ layer from patient tissue biopsies with the advent of induced pluripotency technology (Takahashi and Yamanaka, 2006). This technological development was extremely beneficial to researchers in the field of neurodegenerative disease, as it enabled us to finally generate human neural material that could harbour the genetic abnormalities associated with human disease and express disease causing toxic proteins at presumably endogenous levels (reviewed by Wu et al., 2019). To date, disease related phenotypes have been observed in human PSC-derived neurons for Alzheimer's disease (Arber et al., 2019), Parkinson's disease (Arber et al., 2017), and amyotrophic lateral sclerosis (Tyzack et al., 2019); a range of HD lines with different CAG-repeat lengths have also been used to study HD, many of which were isogenic, or genetically matched (Camnasio et al., 2012; Conforti et al., 2018; Garcia et al., 2019; HD iPSC Consortium,
2017; HD iPSC Consortium et al., 2012; Mattis et al., 2015; Xu et al., 2017b), thereby confirming these can be a useful model for future studies into neurodegenerative disease. With that said, genetic heterogeneity and sub-optimal differentiation protocols can produce spurious results in culture, therefore any disease phenotypes revealed in PSC-derived cultures must be interpreted with caution, and where possible, compared to genetically related, or isogenic controls. More than that – studies have shown that genetic heterogeneity and differences in culture techniques are by far and away the biggest driver in terms of phenotypic differences observed (Volpato et al., 2018).

1.8.3 Human models

The ideal scenario to study human neurodegenerative disease, would be to sample brain material from living patients at repeated time points, so that a longitudinal analysis of disease pathology and progression could be completed. This however is not feasible due to ethical and practical reasons, confounded also by the large number of subjects that would be required to properly power such studies. We can, however, obtain non-invasive samples from living patients to be converted into stem cells by inducing pluripotency, or genetically manipulating already harvested embryonic stem cells. Indeed, PSC resources have come to the forefront of modelling neurodegenerative diseases including HD, as they enable us to investigate early events in disease pathogenesis, as well as follow the development of pathology and disease progression in real-time (Tousley and Kegel-Gleason, 2016; Wu et al., 2019).

1.8.3.1 PSCs

Over the past decade, a flood of differentiation protocols have been published that can reliably produce cells from the three germ layers, including neurons, in an in vitro format (Arber et al., 2015a; Carri et al., 2013; Shi et al., 2012; Victor et al., 2014). By reprogramming somatic cells taken directly from patients, such as HD gene carriers, and reprogramming them to pluripotency prior to differentiation, we can be sure that the endogenous mutation carried by that patient is also present in the cellular material generated (Wu et al., 2019). Furthermore, it is possible to genetically manipulate these PSCs further, by for example 'editing out' the precise disease causing mutation, thus generating an isogenic control for comparison (Cong et al., 2013; Mali et al., 2013).

Since the development of MSN and CPN differentiation protocols, many publications have been produced which explore the HD-associated phenotypes that emerge in culture (Castiglioni et al., 2012; Conforti et al., 2018; HD iPSC Consortium, 2017; HD iPSC Consortium et al., 2012; Mattis et al., 2015; Mehta et al., 2018; Nekrasov et al., 2016; Wiatr et al., 2018). These will be discussed in detail throughout this thesis.

1.8.3.2 Neurodevelopmental biology

The development of the brain is described as the most complex developmental process that occurs through gestation, and continues postnatally throughout childhood and adolescence (Stiles and Jernigan, 2010). All neural material stems from epiblast cells which migrate to the rostral region of the primitive streak on day 13 (E13) of gestation. These epiblast cells form into
the three gestational layers: endoderm, mesoderm and ectoderm, the latter of which is responsible for forming both the peripheral organs such as skin (epidermal ectoderm) and the brain and central nervous system (neuroectoderm). The signalling of TGF-β family members such as BMP and Noggin are inhibited causing neural induction and the differentiation into neural progenitor cells – these are the starting source for all neuronal tissue (Meyers and Kessler, 2017). Neurodevelopment is coordinated by a series of signals which emanate, in part, from the primitive node (Stiles and Jernigan, 2010). This molecular signalling centre induces a change in gene expression in cells migrating along the rostro-caudal axis of the embryo, causing them to secrete TGF-β inhibitors which catalyses the differentiation of underlying epiblast cells into neural progenitors. Over time, the signals generated by the primitive node alter, causing a rostro-caudal organisation to form; forebrain structures such as the cortex and subcortical regions are formed from the earliest migrating cells (rostral), whilst later migrating cells will form hindbrain structures (caudal) (Budday et al., 2015; Stiles and Jernigan, 2010).

The second stage of neurodevelopment is the formation of primary vesicles which occurs immediately prior to neural tube closure. The prosencephalon is particularly important to this thesis as it is the primary vesicle from which both CPNs and MSNs will eventually be derived, after its differentiation into the telencephalon and diencephalon.

The different structures within the brain are formed as a result of molecular patterning orchestrating a multitude of gradients throughout the brain. For example, opposing expression levels of the transcription factors Emx2 and Pax6 orchestrate an anterior lateral to posterior medial gradient which aids the formation and separation of motor cortex, and visual cortex neural progenitor cells (Budday et al., 2015; Meyers and Kessler, 2017; Stiles and Jernigan, 2010). Neural progenitors migrate along these gradients receiving further extrinsic cues ultimately guiding them towards a specialised and terminal cell fate.

**Striatal Neurodevelopment**

As aforementioned, the striatum and other structures that make up the basal ganglia are derived from the ventral telencephalon that forms the lateral ganglionic eminence (LGE) (Arber et al., 2015a; Campbell, 2003). Developmental biologists have capitalised on the understanding of this sequential process, to produce protocols that mimic the sequence of events and extrinsic cues that result in striatal neurodevelopment. As such, we are now able to produce striatal neurons from PSCs and other sources.

Successful striatal differentiation begins with a period of neural induction, which can be achieved by dual SMAD inhibition. SMAD inhibitors such as SB431542, dorsomorphin and LDN-193189 act to inhibit TGF-β and BMP, resulting in induction to neuroectodermal cell fate (Arber et al., 2015a; Carri et al., 2013). Dependent on the protocol in question, a variety of different extrinsic cues can then be used to induce ventral telencephalic fate - thus producing LGE regionalised cells - including activin A and sonic hedgehog (SHH) signalling modulation.
Activin A is a member of the multifunctional TGFβ protein family and has been described as a critical protein required for neurogenesis (Abdipranoto-Cowley et al., 2009). In 2012, it was identified that inclusion of activin A within differentiating neuroectodermal cultures could induce a caudal ganglionic eminence (CGE) fate, the cells of which went on to develop cortical interneurons (Cambray et al., 2012). It was hypothesised by the Li group that application of activin A at an earlier time point within differentiation could induce regionalisation to an LGE fate. This hypothesis was proven correct in 2015 by the same group, in which their activin-mediated differentiation protocol produced functionally active MSNs within 36 days (Arber et al., 2015a). Investigation into the mechanism of action produced a link between activin A exposure and expression of the transcription factor CTIP2, whereby longitudinal activin exposure resulted in a consistent increase in CTIP2-expressing neurons (Arber et al., 2015a).

In contrast, modulation of the compounds Wnt or SHH act to achieve the same result of regionalising the culture, to the ventral fate necessary to develop striatal neurons. This regionalisation can be achieved by exposing neural progenitor cultures to a higher concentration of SHH, or alternatively by inhibiting Wnt signalling by exposing cultures to dickkopf 1 (DKK1), in the presence of a low SHH concentration (Li et al., 2009). The reason for this is that both of these techniques act to modulate the expression and activity of zinc-finger protein GLI3, which normally acts to modulate SHH signalling. This DNA-binding transcription factor is expressed in both full-length and truncated forms and is principally modulated by Wnt signalling. Wnt acts to increase the expression of full-length GLI3 resulting in antagonisation of SHH signalling and a dorsal regionalisation. However, when SHH concentration is increased, or Wnt signalling is inhibited, the truncated form of GLI3 is preferentially expressed and SHH signalling is thus increased, leading to ventralisation of the culture (Li et al., 2009).

Once ventralised, striatal progenitors can be matured into functionally active MSNs by the application of several growth factors, including BDNF and GDNF, combined with exposure to retinoic acid (RA), the bioactive form of vitamin A. RA is required for the proliferation, differentiation and homeostasis of diverse cell types, and although not essential in early stages of differentiation, it is required at later stages to produce terminally differentiated, functional striatal neurons (Podleśny-Drabiniok et al., 2017). Similarly, the growth factor BDNF is required to facilitate MSN maturity by inducing transcriptional changes resulting in reduced apoptosis and dendritic spine development. Finally, GDNF is also a neurotrophic factor which although not critical for MSN survival, has been suggested to contribute to the further fate specification of MSNs, inducing the indirect pathway specific neurotransmitter substance P (Humpel et al., 1996).

1.8.3.3 Neurodevelopmental biology of the cerebral cortex

Contrary to striatal neurodevelopment, no further chemical stimulation is required to produce cortical neurons once cultures have undergone neural induction. This is because the default patterning of neural progenitors once successfully neurally induced, is to pattern to the layers of the cerebral cortex. Whilst ventralisation is required to produce striatal neurons, maintained Wnt signalling specifies dorsal regionalisation of the telencephalon, resulting in cortical patterning.
Development of the six distinct layers of the cortex however does require external guidance in the form of radial glial cells. The formation of the cortical plate is dependent on the migration of cortical progenitors along radial glial cells from ventricular and subventricular zones. In the early stages of cortical plate development, two ‘zones’ are established: a superficial marginal zone, and the subplate. Cortical progenitors accumulate in layers above the subplate to form the distinct layers of the cortex, with early-born neurons establishing the deepest layers of the cortex – layers 5 and 6 – whilst later born neurons migrate towards the marginal zone to form the more superficial layers; the marginal zone eventually becomes cortical layer 1 (Budday et al., 2015). Once a cortical neuron reaches its terminal destination, it can begin to form connections with intended targets. As deeper-layer neurons are born first, they can be distinguished not only by their location within the cortical plate, but also by their morphology as they possess elaborately branched dendritic trees accompanied by a rounded cell body. This is in contrast to newly born neurons which posses an apical dendrite, elongated cell bodies and a descending axon which remains connected to the subplate (Bystron et al., 2008).

If the order and timeline of events outlined above is recreated in vitro, as demonstrated by (Shi et al., 2012), it is possible to generate CPNs from PSCs. The length of time neurons are maintained in culture will determine how enriched the culture is for a specific cortical layer. Earlier terminated cultures will be enriched for the deepest layer neurons, whilst any extension to culture period will produce proportionally more upper layer neurons.

By following the above described procedures, researchers have been successful in generating MSN and CPN cultures derived from both healthy and disease cell donors, enabling the investigation of early events in disease pathogenesis, and contributing to our knowledge of previously unknown developmental defects that may be occurring in neurodegenerative disease.

1.9 Existing models of the CS pathway

Previous studies into the disrupted CS connectivity in HD have been conducted primarily in animal models of the disease, although imaging data has also been produced to support this hypothesis in human patients (McColgan et al., 2015). More recently, efforts have focused on attempting to recapitulate the CS pathway in vitro to enable study on a level in which cellular isolation is possible.

1.9.1 Animal models

Studying freely-moving animals i.e. non head-fixed, that have intact brain circuitry, as well as brain slices obtained from HD animal models at defined disease time points, has increased understanding of the electrophysiological abnormalities that occur in the CS pathway during HD. In transgenic models of HD, multiple groups have observed an increased firing rate of MSNs at rest, with concomitant decreases in burst firing and decreased synchrony in firing across networks, suggesting that MSNs are hyperexcitable, but firing is not co-ordinated or regulated as it should be (Bunner and Rebec, 2016; Estrada-Sánchez et al., 2015; Miller et al., 2011; Walker et al., 2008). Unsurprisingly, evidence for dysregulated glutamate signalling as well as
dopamine modulation is well documented in the literature. In R6/1 mice, NicNiocaill et al., identified a prolonged glutamate presence in the synaptic cleft which was caused by a rapid release of glutamate, followed by a delayed re-uptake in the striatum (NicNiocaill et al., 2001). When assessing dopamine levels in the brains of transgenic HD mice, Dallérac et al., found a dramatically increased concentration of dopamine in the brains of pre-symptomatic mice, contrasting with a drastic reduction of the neurotransmitter in later stages of this disease. This combined with the opposing roles of dopamine on the direct and indirect pathway MSNs - inhibiting D2-expressing MSNs (indirect pathway) yet exciting D1-expressing MSNs (direct pathway) - is another piece of supporting evidence explaining the progression from hyperkinetic to bradykinetic movement during the course of the disease (Dallérac et al., 2015). Finally, in freely moving transgenic animals, researchers have been able to acquire whole network activity data from both R6/1 and R6/2 mice (Cabanas et al., 2017). During a procedural learning task, Cayzac et al., identified that the R6/1 striatum was not 'recruited' or activated to the same level as control mice (Cayzac et al., 2011). In contrast, striatal activity at rest in R6/2 mice was recorded as higher than WT littermates by Rebec, Conroy and Barton, a phenomena that was discovered to be due to reduced ascorbate levels in the extracellular space of HD mouse brains - upon treatment with ascorbate, the activity levels reverted to WT levels (Rebec et al., 2006).

1.9.2 Human imaging data

By using advanced neuroimaging techniques, it has been possible for researchers to visualise the CS pathway in human patients. By applying complex analytical software, such as tractography, we are now able to detect changes in connectivity levels between brain areas and compare them in diseased and healthy brains. Several studies have been conducted that focussed on the connectivity between cortical and striatal regions all of which found abnormalities when compared to control subjects. Marrakchi-Kacem et al., analysed the brain scans of symptomatic HD patients and controls prior to applying high angular resolution diffusion-weighted data (HARDI) to recover the anatomical connectivity between regions. Their results indicated that a reduction in the number of tracts connecting the striatum and cortex were evident in HD patients. The connectivity between regions differed in magnitude depending on if the caudate or putamen was assessed: caudate preferentially lost connections with parietal over frontal regions, whereas the putamen loss connectivity preferentially with temporal, then frontal, then parietal regions (Marrakchi-Kacem et al., 2013). Novak et al., identified altered structural connectivity between the cortex and striatum, in which the putamen, pallidum and thalamus were most affected (Novak et al., 2015). McCollgan et al., used MRI and diffusion tractography analysis to assess premanifest and manifest HD patients against control subjects and also noted altered cortical connectivity to the caudate nucleus, putamen and the thalamus. Both the caudate and putamen also showed abnormal connectivity to their onward targets and the degree of this loss appeared to correlate with the total motor score of patients upon assessment. The authors hypothesised that the breakdown of long range connections, such as the CS pathway, in premanifest patients caused a network segregation to occur between brain regions, which progressed to a loss of integrated connectivity upon symptom manifestation (McColgan et al., 2015), a finding that was echoed in a study conducted by Poudel et al., in
which the authors suggested that altered synchrony across neural networks, including altered CS connectivity, would lead to the development of clinical symptoms in patients (Poudel et al., 2014).

1.9.3 Primary cell models

In vitro models of the CS pathway using primary neurons isolated from HD mouse models have contributed to our knowledge and understanding of how this pathway is affected in HD. Most models to date have used a mixed population paradigm, in which cortical and striatal neurons are dissected in isolation, prior to mixing cell suspensions and seeding on the same coverslip. Non-contact models have also been designed in which mono-cultures are seeded prior to placement in a communal culture device, as well as the utilization of non-permeable substances such as wax or cell-culture inserts. Relatively recently, more sophisticated culture systems have been developed which enable culture compartmentalisation, these are constructed using microfluidic devices.

1.9.3.1 Mixed population

The concept of mixed population models is such that CPNs and MSNs are in direct contact with each other, sharing communal media and free to form as many or as little synaptic connections as required by the culture. A wide array of data has been collected by multiple research groups detailing HD deficiencies in CS co-cultures, largely pertaining to electrophysiological abnormalities, dendritic tree complexity and neuronal viability. In 2012, Milnerwood et al., developed mixed population co-cultures derived from WT and YAC128 mice. Focusing primarily on electrophysiology and glutamatergic signalling, the authors identified that YAC128 cultures had an increased extrasynaptic NMDAR current. These results were found to be cell autonomous with regards to MSNs, as in chimeric cultures the phenotype was evident when YAC128 MSNs were used, irrespective of pre-synaptic cortical phenotype (Milnerwood et al., 2012). With the advent of optogenetic techniques, Artamonov et al., were able to selectively stimulate WT and YAC128 CPNs co-cultured with MSNs and observe activity differences. By stimulating CPNs and recording from MSNs, the authors identified a disrupted synaptic transmission in YAC128 cultures (Artamonov et al., 2013).

Consistent with these data were reports from firstly Buren et al., followed by Schmidt et al., in 2018, in which the same experimental design was used with WT cultures and YAC128 cultures, respectively. In these studies, the authors manipulated the ratio of cortical: striatal seeding density; either 1:1 or 1:3 cultures were seeded in mixed population coverslips. Strikingly, similar results were seen between WT and YAC128 studies, in which the lower cortical plating ratio (1:3) exhibited reduced striatal viability, reduced cell capacitance, a reduction in dendritic arborisation complexity and total dendritic length. However, in the latter study, phenotypes were also observed in the equal plating ratio (1:1), including reduced dendritic complexity and overall dendritic tree length, as well as the finding of reduced dendritic spine number in 1:3 YAC128 cultures. This study also constructed chimeric cultures which identified that these phenotypes were MSN cell autonomous, as whenever YAC128 MSNs were present in the culture, the
phenotypes emerged, including a reduced viability phenotype after 21 days in culture (Buren et al., 2016a; Schmidt et al., 2018).

Finally, a more recent study conducted by Yu et al., in 2018 assessed different aspects of BDNF such as release potential and trafficking in the zQ175 knock-in HD mouse model. The authors found that in both isolated CPN cultures, and those co-cultured with MSNs, there was a decreased concentration of BDNF released in HD cultures and BDNF trafficking was significantly reduced (Yu et al., 2018).

1.9.3.2 Microfluidic models

To enable easier analysis of directionality dependent phenotypes, as well as examine constituents of co-cultures in isolated environments, there has been a movement toward using compartmentalised culture devices such as microfluidic chambers (MFCs), which are particularly useful for studying neuronal phenotypes in neurodegenerative disease (Fantuzzo et al., 2019).

MFCs are specialised culture devices that consist of two or more chambers, usually connected by micro-channels (Figure 1. 8A) Explained in more detail throughout the thesis, MFCs enable the researcher to limit physical interaction between cell populations, as well as introduce fluidic isolation to the culture if desired (Figure 1. 8C). As such, therapeutic treatments can be applied to one cell type for example, and read-outs acquired from a second. The devices lend themselves perfectly to studying neuronal populations, as whilst the micro-channels prevent free movement of cell bodies between compartments, the micro-channels permit axon projection (Figure 1. 8B). There are very few published reports using MFCs to construct the CS pathway in a HD context, however those in the literature have proven informative.

Figure 1. 8. Microfluidic chamber (MFC) design and properties. MFCs are culture devices constructed of two or more chambers (A) in which cells can be seeded and thus physically separated. Micro-channels (B) connect the two chambers, lending themselves to studies assessing neuronal cells as the micro-channels are amenable to axon projection. The chambers can be fluidically isolated from each other (C) as shown by the separation of blue and red food colouring.

Zhao et al., assessed synapse formation and aspects of BDNF functionality in co-cultures derived from primary BACHD neurons seeded in MFCs. In contrast to the previous results described above, the authors identified a reduction of synapses in co-cultures that contained
BACHD CPNs, irrespective of MSN genotype, suggesting that mHTT expression in the presynaptic neuron was sufficient to initiate disease pathology (Zhao et al., 2016).

The most complex recapitulation of the CS pathway to date was published by Virlogeux et al., in 2018. This study developed a tri-chamber MFC, technical and experimental details of which will be discussed in Chapter 5. Once established, the authors analysed BDNF anterograde and retrograde transport, and reported the same findings as discussed previously; both anterograde and retrograde transport of BDNF was compromised in HD co-cultures. The authors also constructed chimeric cultures in a similar format to above, and identified that the phenotypes described were dependent on the genotype of the presynaptic compartment i.e. the CPNs. In cultures where CPNs expressed mHTT, the HD-associated phenotype was observed, irrespective of the genotype of MSNs, thus supporting the finding by Zhao et al (Virlogeux et al., 2018).

1.9.4 Caveats of existing models

It goes without question that there are caveats to the models described above, which limit the translatability of the results. In mixed population models, there is no structure or organisation to the connections formed, thus although cortico-striatal synapses will indeed be formed, anatomically incorrect striato-cortical synapses may also be formed. Furthermore, due to the high intraneuronal connectivity, these striato-cortical synapses may alter CPN behaviour and thus confound the identification of HD-associated phenotypes. There is the possibility of auto-synapsing i.e. axo-axonal as well as axo-somatic synapses, forming in these cultures, again due to the unorganised cell seeding. Whilst in the broader context of HD it may be interesting to evaluate the impact of MSNs on CPN activity, when considering the CS pathway individually, this information is superfluous, as it is not physiological relevant. Another caveat of mixed population models of the CS pathway, is the difficulty posed in confirming the identity of the neuron from which you are recording. Although the majority of the studies described above utilised transient transfection of fluorescent proteins prior to mixing cell suspensions, the transfection efficiency is very unlikely to be 100%, and very few studies use retrospective dye labelling (Arber et al., 2015b). Distinguishing between neuronal populations based on morphology is challenging even in sparse cultures, as such it cannot be relied upon in co-culture cases in which hundreds of thousands of cells have been seeded. Taken together these caveats may be cause for concern, as one cannot be 100% certain of the cellular identity one is observing or recording from.

The latter caveats discussed here, are negated when considering MFCs, as the physical separation of seeding chambers ensures that the researcher can confirm the identity of the neuron or sample in question. Overall, these devices introduce a much higher level of specificity and control over co-cultures, as almost every aspect of culture can be controlled including if the media within each compartment must be fluidically isolated for example. The main caveat of these devices however, relates to this; the size limitations of the devices themselves mean that a relatively small number of neurons can be seeded, in order not to overwhelm the chambers.
and result in channel blockage. Furthermore, depending on the fragility of the neurons in question, the maintenance of the devices could be challenging; repeated media changes into a small, constricted chamber introduces large pressure changes and exerts shear stress on the cells. With that said, these devices are thus far the best platform in which neural circuitry can be modelled both biologically and physiologically in vitro.

The final caveat that pertains to both these models, is that so far in the literature, they have only been completed with murine cells. Indeed, to date there are no publications of the use of human cells in MFCs in the context of HD, or even in mixed population co-cultures (Fantuzzo et al., 2019). Secondly, with regard to human studies, they have investigated HD-associated phenotypes in only one of the primary cellular targets of HD - either human PSC-derived MSNs, or CPNs. As of yet, there have been no studies that have looked at both cell types in combination. Hence, to date there has not been a human recapitulation of the CS pathway in vitro, which led to the conception and design of this project.

1.10 Thesis aims

1.10.1 General research goals

The overarching goal of my PhD was to design and optimise a platform in which the CS pathway could be reliably recapitulated using human PSC-derived neurons. I aimed to establish both pure and ‘chimeric’ cultures to enable investigation into the exact driver of the pre-synaptic and post-synaptic pathology, as well as complete these co-cultures with a range of CAG-repeat lengths to identify if CAG-repeat length impacted upon co-culture phenotype.

1.10.2 Specific aims and objectives

1) Differentiate and characterise PSC-derived MSNs and CPNs from iPSCs generated from an HD family and an isogenic ESC HTT allelic series:
   i) Adapt the Arber et al protocol to reliably produce MSNs with CAG-repeats ranging from 22Q - 81Q in a standardised manner;
   ii) Adapt and optimise the Shi et al protocol to reliably produce layer V CPNs with CAG-repeats ranging from 22Q - 81Q in a standardised manner;
   iii) Develop novel protocols for the cryopreservation and thawing of partially differentiated MSNs and CPNs, to facilitate experimental designs requiring synchronised differentiations;
   iv) Characterise PSC-derived neurons using immunofluorescence to confirm cellular identity.

2) Evaluate and quantify the emergence of HD mutation-associated phenotypes in PSC-derived MSNs and CPNs:
   i) Assess key parameters such as viability and adhesion over a time course;
   ii) Identify the impact of exogenous cellular stress on neuronal viability (MSN specific);
   iii) Assess the maturation of PSC-derived neurons using electrophysiological techniques (MSN specific);
iv) Measure the expression of BDNF in PSC-derived neuronal cultures and compare expression levels and release (CPN specific).

3) Design and optimise a co-culture platform that biologically and physically recapitulates the CS pathway:
   i) Establish mixed population co-cultures of PSC-derived CPNs and MSNs in a shared culture device;
   ii) Trial the seeding and maintenance of PSC-derived CPNs within in-house generated MFCs;
   iii) If ii) successful, trial the seeding and maintenance of PSC-derived MSNs in MFCs;
   iv) If ii) and iii) successful, design a seeding and maintenance protocol to co-culture PSC-derived CPNs and MSNs in MFCs;
   v) If all of the above is successful, generate pure (WT:WT, HD:HD) and chimeric (WT:HD HD:WT) co-cultures in MFCs and assess connectivity. Ideal measures of assessment: cell viability, synapse formation, synaptic transmission, BDNF trafficking and release within a co-culture setting.

Chapter 2 – Materials and methods

2.1 Reagent preparation
All reagents used for cell culture were prepared as per the manufacturer's instructions and diluted to the appropriate final concentration as found in Table 3. Where appropriate, reagents were sterile filtered, and aliquoted to avoid freeze-thaw. Unless stated otherwise, culture reagents were sourced from Gibco. The cell culture media was made up as follows:

**MSN pre-26 media** = 3:2 mixture of DMEM/F12 and Neurobasal, 1x N2, 1x B27 (without vitamin A), 200 mM L-glutamine, 100 μM 2-mercaptomethanol.

**MSN post-26 media** = 3:2 mixture of DMEM/F12 and Neurobasal, 1x N2, 1x B27 (with vitamin A) 200 mM L-glutamine, 100 μM 2-mercaptomethanol, 50 U/mL penicillin, 50 mg/mL streptomycin (50 mg/ml Pen/Strep).

**N2B27 media** = 1:1 mixture of N2 medium [DMEM/F12 GlutaMAX, 1x N2, 5 μg/mL insulin, 1 mM L-glutamine, 100 μM nonessential amino acids, 100 μM 2-mercaptomethanol, 50 U/mL pen, 50 mg/mL strep] and B27 medium [Neurobasal, 1x B27, 200 mM L-glutamine, 50 U/mL pen, 50 mg/mL strep].
Table 3. List of materials and reagents used for PSC maintenance, MSN and cortical differentiation and maturation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration MSN/CPN (italicised)</th>
<th>Cat. no</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F-12 (w/o glutamine)</td>
<td>--</td>
<td>21331-046</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>Neurobasal</td>
<td>--</td>
<td>21103-049</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>D-PBS</td>
<td>--</td>
<td>14190-094</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>Distilled Water</td>
<td></td>
<td>15230-188</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>N2</td>
<td></td>
<td>17502-048</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>B27 (w/o vitamin A)</td>
<td></td>
<td>12587-010</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>B27 (with vitamin A)</td>
<td></td>
<td>17504-044</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDN193189</td>
<td>100 nM</td>
<td>SML0559</td>
<td>Sigma</td>
</tr>
<tr>
<td>SB431542</td>
<td>10 μM/10 μM</td>
<td>SM33-10</td>
<td>Cambridge Biosciences</td>
</tr>
<tr>
<td>Dorsomorphin</td>
<td>200 nM/1 μM</td>
<td>SM03-10</td>
<td>Cambridge Biosciences</td>
</tr>
<tr>
<td>Activin A</td>
<td>25 ng/mL</td>
<td>AF-120-14E</td>
<td>PeproTech</td>
</tr>
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<td>BDNF</td>
<td>10 ng/mL</td>
<td>AF450-02</td>
<td>PeproTech</td>
</tr>
<tr>
<td>GDNF</td>
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2.2 Human pluripotent stem cells (PSC)

The CAG-repeat length within the HTT gene encodes for a string of glutamine residues, commonly referred to as polyQ, however at the end of the CAG-repeat, there is a CAACAG terminus, both of which encode glutamine. Therefore, although a persons’ HTT gene may be composed of 50 CAG-repeats, the polyQ length should technically be reported at 52, due to the two additional glutamine residues. Within this thesis, the lines used are described as both XX-CAG repeats, as well as XXQ, therefore the number differs between descriptions. To clarify, a control line expressing 20 CAG repeats, will be referred to as 22Q.

2.2.1 Generation of PSCs

For the HD family induced PSC series (iPSC), fibroblast samples were kindly donated from an HD family, comprising an unaffected parent (20 CAG repeats within the HTT gene), and three offspring with juvenile HD, with CAG-repeat lengths of 56/20, 67/20 and 73/20. The fibroblasts were reprogrammed in a non-integrated manner to iPSCs by Sendai reprogramming (CytoTune-IPS 2.0 Sendai Reprogramming kit, Life Technologies, A16517, A16518) by collaborators at the Rockefeller University (Prof Ali Brivanlou). As depicted in Figure 2. 1, three clones were obtained for each donor (line), allowing us to control for any variability caused by the reprogramming itself. For this project, experiments were conducted primarily with the three clones containing the longest CAG repeat length (75Q clone 1/2/3), compared to the three 22Q clones (22Q clone 1/2/3) unless stated otherwise. This series is from now on termed the HD family series.

To complement and corroborate the results generated from the HD family series, experiments were repeated with an isogenic allelic series of human ESCs generated and kindly shared by Dr Mahmoud Pouladi, (A* Institute, Singapore University). Briefly, the female human ESC line [H9] was subjected to genome editing using TALENs whereby the CAG repeat sequence in exon 1 of the HTT gene was modified to include 28, 43, 63 or 79 CAG-repeats (Figure 2. 1). As the control line (30Q) was also subject to modification, the impact of the genome editing process was controlled for. Multiple clones of each isogenic line were then generated, however this project used a single clone of each CAG-repeat length for experimentation in multiple differentiations. This series is from now on termed the IsoHD series.

2.2.2 Thawing PSCs

Six-well culture dishes were coated with Geltrex® hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Gibco) diluted 1:100 in cold DMEM: F12, and incubated at 37°C for 1 h. Cryopreserved PSCs were gently thawed by partially submerging in a 37°C water bath for 30 s - 1 min until an ice crystal remained. The contents were gently transferred dropwise to a 15 mL Falcon centrifuge tube using 5 mL stripette on aspiration level one, before 7 mL room temperature (RT) Essential 8 (E8) media was added drop-wise. The Falcon was inverted every 2 mL to aid gentle mixing in with the fresh medium and reduce osmotic shock to the cells. The cells were centrifuged at 200xg for 5 min, supernatant was discarded and the cells were re-
suspended in 2 mL fresh E8 media. Geltrex was aspirated before the cell suspension was added dropwise to the wells. One cryovial was plated into a single well. Cells were distributed across the well by moving the plate laterally in a ‘cross formation’ and incubated at 37°C [5% CO₂/air] for 24 h before a full media change was completed to remove cell debris.

2.2.3 Maintenance of PSCs

PSC cultures were maintained on Geltrex substrate diluted as previously described, in E8 media which was changed every 24 h. To maintain cells for more than 24 h, cultures were changed to double volume Essential 8 Flex media, which can sustain cultures for two days. When PSC colonies reached 80-90% confluence they underwent an EDTA passage as described below.

Upon reaching confluency, PSC colonies were passaged at 1:10 (Monday) and 1:6 (Friday) ratios, subject to experimental timeline. The Geltrex was aspirated, and replaced with 1 mL RT E8 media. The media was discarded and cells were washed once in PBS, before 1 mL 0.5 mM EDTA (warmed to 37°C) was added to the cells and incubated for 4 min. The EDTA was aspirated and using a 5 mL stripette, cells were washed off the plate with 1.5 mL E8 media using a pipette boy on eject level three. A maximum of three washes were used to remove cells, before they were diluted appropriately and added dropwise to the recipient plates.

Quality control experiments were conducted on both PSC cohorts prior to use for differentiation and experimentation. These experiments involved routine and repeated mycoplasma testing, CAG-repeat sizing and karyotyping. Experiments were also conducted assessing the pluripotency of the cells post-reprogramming by assessing the expression levels of key pluripotency markers Oct4, Sox2 and NANOG. These experiments were completed prior to the start of this project and are therefore not included in this thesis.

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<th>Disease State</th>
<th>CAG length</th>
<th>ESC clone generated</th>
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<td>HD</td>
<td>79</td>
<td>81Q</td>
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</table>

Figure 2.1. PSC donor demographics. The HD family series comprised three clones of an unaffected parent [22Q (1/2/3)], and two offspring who developed juvenile HD [58Q (1/2/3), 75Q (1/2/3)]. The IsoHD series comprised lines expressing 28, 43 and 78 CAG repeats. Clones marked in bold are those selected to generate the data presented in this thesis. Figure adapted from (Park et al., 2008).
2.3 Generation of PSC-derived medium spiny neurons (MSNs)

For this project, mature MSNs were produced using an adapted version of an established protocol (Arber et al 2015). The protocol uses the combination of SMAD inhibition, followed by activin A and vitamin A stimulation over a period of 36 days to generate a heterogeneous neuronal culture. Within this population, a proportion of cells can be identified as MSNs due to their co-expression of DARPP-32+/CTIP2+.

2.3.1 Neural induction

As depicted in Figure 2, the adapted protocol ran as follows. Following several passages, and consistent stable colony formation (circular colonies with increasing diameter, no evidence of contaminating cells or spontaneous differentiation), PSCs were seeded into Geltrex coated 12-well plates at high density; typically, a fully confluent well of a six-well plate was split into three wells of a 12-well plate using the standard PSC EDTA passage procedure. PSCs were cultured to approximately 90% confluency at which point neural induction was initiated. Cells underwent a full media change into MSN pre-26 media, supplemented with the following SMAD inhibitors: 100 nM LDN193189, 10 μM SB431542, 200 nM dorsomorphin. SMAD inhibition rapidly downregulates pluripotency transcripts such as OCT4 and NANOG, causing the cells to differentiate towards forebrain neural precursor fate of the neuroectoderm lineage. Neural induction was continued for 10 days, with cells undergoing a half media change every 48 h.

Figure 2. Summary of medium spiny neuron (MSN) differentiation protocol. Over the period of 36 days, PSCs were differentiated to lateral ganglionic eminence precursors and matured to MSNs. The cells underwent sequential media supplementation changes as well as different plate coatings to encourage neuronal differentiation and maturation. These were compounded by timed passages at days 10, 19-22 and 30.
2.3.2 Neural precursor cell (NPC) differentiation

At day 10, cells were prepared for their first passage by a complete media change into 1.5 mL MSN pre-26 medium supplemented with 25 ng/mL activin A and 10 μM ROCK inhibitor for a minimum of 1 h. During this time 12-well plates were coated with 25 μg/mL fibronectin in PBS, which were washed once in PBS prior to use. After incubation, the conditioned media was collected from the cells and retained. The cells were washed once in PBS before 0.02% EDTA was added and incubated at 37°C for 1 min. This served to loosen the attachment of cells to the Geltrex substrate to facilitate removal, however no observable difference was noted in the culture. The EDTA was removed and replaced with 1 mL conditioned media, before the cells were scratched gently with a 10 mL stereological pipette to generate large clumps of cells. These were diluted with the remaining conditioned media, followed by fresh MSN pre-26 media including above supplements to a final volume of 1.5 mL per well of a 12-well plate. The final passage ratio was 2:3. Cells were then left to adhere for 24 h upon which 1 mL of media was replaced with 1.5 mL MSN pre-26 media supplemented only with 25 ng/mL activin A, with half media changes continued every 48 h. This acted to gradually reduce the concentration of ROCK inhibitor in the media.

After 19-22 days in culture, the cells had completed neural differentiation and were thus termed neural progenitor cells (NPC). Due to the addition of activin A, the NPCs were directed down the lateral ganglionic eminence lineage, which can be demonstrated by an upregulation of transcripts: CTIP2, FOXP2, DLX2, and GSX2 (Arber et al., 2015a). The NPCs were subjected to a second EDTA passage, in which cells were washed once in PBS prior to addition of 0.02% EDTA and incubated at 37°C for 90 s. The EDTA was aspirated and 1 mL MSN pre-26 media supplemented with 25 ng/mL activin A was added per well. The cells were scratched with a P1000 tip and multiple wells pooled in a 50 mL Falcon tube. The final volume of cell suspension was increased to account for 1.5 mL per well of a 12-well plate, at a passage ratio of 1:2 – 1:4 depending on how confluent the NPCs were (typically 1:3). Passaged cells were re-plated into 12-well plates that had been incubated with 20 ng/mL laminin for a minimum of 2 h, which was aspirated immediately prior to cell plating. The NPCs underwent half media changes every 48 h. At this stage in the protocol, the first adaptation was made as there was an opportunity to pause differentiation and cryopreserve NPCs.

2.3.3 Cryopreservation of NPCs

Two methods of cryopreservation were designed and optimised, one of which utilises StemPro Accutase to lift the cells off the substrate to generate a single cell suspension similar to the ‘Accutase split’ detailed in Shi et al., (2012). The second follows the second passage procedure described above; this freezes cells as small clumps and over the course of this project gave a better revival percentage, therefore was the preferred method. In both protocols, once dissociated from the culture plastic, 1 mL cell suspension was aliquoted per cryovial which contained 100 μL DMSO (final concentration 10% DMSO, 90% MSN pre-26 media). The cryovials were inverted once to ensure thorough mixing prior to placing in a Mr Frosty container, and stored at -80°C for 24 h. Cryovials were transferred to LN2 for long-term storage.
2.3.4 Maturation and seeding
When NPCs reached d26, the cells underwent a full media change to MSN post-26 media, which contains vitamin A and promotes the maturation of NPCs to MSNs. Furthermore, it was observed that maturation was also enhanced when cells were less densely populated in the culture dish, therefore an adaptation to the protocol was made which allowed for better control over plating density and distribution. MSNs were subjected to a third passage, in which Accutase was pre-warmed to 37˚C prior to use (which was found to greatly increase the survival of cells post-passage as it decreased the required incubation time to approximately 5 min) and Geltrex was used as the coating substrate of coverslips or imaging plates to enhance the distribution of the cells when cultured for extended periods of time. After 36 days in culture, the protocol was complete and a population of MSNs could be identified within the heterogeneous culture. For this project, MSN cultures were maintained beyond d36 to encourage further maturation of the neuronal population, and to study the effect of ageing on HD phenotype emergence.

2.3.5 Ageing cultures
To allow for the analysis of maturing cultures, a protocol was developed to minimise stress to the cells. Half media changes were performed twice weekly, approximately every four days. This involved maintaining the plate in a horizontal position, and using a pipette boy on aspiration level two to remove half the media from the well. Fresh media was applied on eject level zero down the side of the well, with the stripette positioned against the very top edge. This acted to disperse the media laterally across the side of the well surface, thereby reducing the force of fresh media entering the well. This prevented a focal point from forming at the cell layer, and greatly improved maintained cell adhesion to the substrate. In cases where cells did start to detach, the media was spiked with the adhesion substrate (laminin 20 ng/ml, Geltrex 1:50 dilution) as required, but no more than once every fortnight. Cells were routinely maintained and thus aged, in this manner to over 100 days in culture.

2.4 Generation of PSC-derived cortical projection neurons (CPNs)
Similarly to MSNs there are multiple protocols, all of which are well established, that produce functionally active, mature cortical neurons from each of the six cortical layers. The core protocol chosen for this project, published by Shi et al., (2012), is widely used in the field and reliably produces cortical layers I-VI in 130 days.

2.4.1 Neural induction
The protocol is depicted schematically in Figure 2.3. Following several passages and consistent stable colony formation, PSCs were seeded into 12-well plates that had been coated with Geltrex diluted as previously described. Typically, a fully confluent well of a six-well plate could be split into two wells of a 12-well plate using a standard PSC EDTA passage procedure. PSCs were cultured to 100% confluency at which point neural induction was initiated. Cells underwent a full media change into N2B27 media, supplemented with the following SMAD inhibitors: SB431542 10 μM, dorsomorphin 1 μM. A 90% media change was conducted every day for 12 days to generate NPCs of the neuroectoderm lineage. During the media change, the
plate was maintained in a horizontal position and the meniscus was unbroken; aspiration of all media proved too harsh on differentiating cultures and resulted in culture death.

Figure 2. 3. Summary of cortical differentiation protocol. PSCs are directed to form neural precursor cells during a process of neural induction, prior to successive neural precursor patterning and differentiation to the six layers of the human cortex in a time dependant manner. Dual SMAD inhibition is sufficient to induce neural lineage, no further media supplementation is required. Plate coatings are altered throughout the 60 days to promote neuronal purity, which is supported by timed passages at d12, d22, d35 and an optional d45-d60 passage.

2.4.2 NPC differentiation

The default patterning of NPCs is to differentiate into the layers of the cortex, therefore no further chemical stimulation was provided to the cells. The original published protocol details a series of dispase splits to purify the cultures of any non-neuronal cells and encourage neural rosette formation. However it was established during the course of this project that by following the EDTA passage method as described in 2.3, the viability of cortical NPC cultures post-passage was greatly increased and did not negatively alter differentiation efficiency or neuronal purity at the termination of culture. Quantitative nor qualitative data was collected prior to culture disposal therefore is not presented in this thesis. Cortical NPCs underwent EDTA passages as described above on days 12 and 22 at ratios 1:2 and 1:3 respectively. Cultures were maintained on laminin coated plates with media changes every 48 h. At this point in culture, neural rosettes were evident, and neural processes began to form networks across the culture dish.

2.4.3 Cryopreservation of cortical NPCs

Similarly to MSN NPCs, differentiating cortical cultures are also amenable to cryopreservation, however this is completed at d30 due to the slower differentiation rate. Cortical NPCs are frozen down using the Accutase passage method as described below. However the cell pellet is re-suspended in ice cold N2B27 media and aliquoted into cryovials, prior to the addition of DMSO solution to a final concentration of 10% before transferral to -80°C in a Mr Frosty freezing container. After 24 hours, cryovials could be transferred to LN₂ for longer term storage.
2.4.4 Deep layer cortical neuron seeding

After 35 days in culture, deeper cortical layer neurons (layer VI evident, layer V emerging) make up the majority of cells in the culture. To seed into experimental format, cells underwent an Accutase passage to generate a single cell suspension. Briefly, media was aspirated and 500 μL pre-warmed Accutase was added to wells and incubated for 5 min. During this time, the cells lift off in the Accutase as a single sheet which can be picked from the wells using a P1000. The cell sheet was dispersed in 10 mL PBS and centrifuged at 350xg for 5 min. The pellet was resuspended in RT N2B27 media and cell density was calculated. The appropriate volume of cell suspension was diluted in N2B27 medium and distributed in laminin coated culture vessels. If there was an excess of cells above what was required for experiments, these were frozen down at a minimum density of 1 x 10^6 cells/vial. As cortical NPCs tended to proliferate at higher rates than MSN NPCs, the cultures were matured at semi-sparse density with twice weekly media changes. As this project was focused on recapitulating the CS pathway, layer V was the primary interest, as this layer contains the cortical neurons that project to the striatum and form the CS pathway. For this reason, cultures were typically maintained and terminated between 50 - 60 days in culture, as more superficial layers of the cortex were not required. Furthermore, it was determined that the CPNs could be subject to further Accutase passages between days 45 - 60 to amplify the percentage of layer V neurons whilst also reducing the number of proliferating NPCs. Cultures could be aged in the same manner as described in 2.3.5 and cultures were successfully maintained to 200+ days in culture.

2.5 Cell imaging

2.5.1 Standard immunofluorescence imaging

Neuronal cells were subject to an Accutase passage and seeded at a density of 3x10^4 per 13 mm diameter glass coverslip in 4-well, or 24-well culture plates (Nunc). Glass coverslips were sterilised and tempered to improve substrate adhesion by baking in an electric oven at 200°C for a minimum of 6 h, and stored in sterile conditions until use. Coverslips were coated with 150 μL Geltrex per coverslip. Immediately prior to seeding, Geltrex was aspirated, no washing was required.

When cells reached maturity, supernatant was aspirated and replaced with 250 μL 10% formalin solution for 15 min at RT. Formalin solution was removed with two washes in PBS, then cells were permeabilised with 0.2% Triton-X 100 (Sigma) in PBS for 15 min. Non-specific binding was blocked with 10% donkey (dk) or goat (gt) serum (dependent on secondary antibody host species), and 1% bovine serum albumin (BSA) in PBS for 1 h at RT, followed by incubation with primary antibodies at the appropriate dilution (see Table 4) for either 2 h at RT, or 4˚C overnight. Primary antibodies were removed and replaced with 300 μL PBS per well and left to incubate for 5 min with no agitation. This process was repeated three times, before Alexa Fluor-conjugated secondary antibodies corresponding to the correct species were diluted 1:1000 in PBS and added to coverslips for incubation in the dark for 45 min. Excess secondary antibody was removed with two washes in PBS, before 1 μg/mL Hoechst in PBS was applied to coverslips for 5 min protected from light. Coverslips were further washed twice in PBS before
mounting onto Superfrost glass microscope slides (VWR) in Antifade Fluorescence Mounting Medium (DAKO) and stored at 4°C until visualisation using fluorescent, confocal or super resolution microscopy.

Table 4. Description of antibodies and the dilutions used within this Thesis

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2.5.2 High content imaging (HCI)

2.5.2.1 Immunofluorescent staining

The seeding density required for HCI differed between cell-type due to the proliferation rates of cells. For CPNs, cells were seeded at 1.5 x 10⁴ cells/well, whereas MSNs were seeded at 2 x 10⁴ into Perkin Elmer Cell Carrier Black 96-well plates (PE plates). Cells were seeded only in the inner 60 wells of each plate, leaving an evaporation barrier of wells around the periphery which could be topped up with sterile media. MSNs were seeded at ~d28 - 30, cortical neurons at ~d35. Once the cells reached the desired age in culture, they were fixed as follows. Using a multichannel pipette, 150 µL culture media was removed and replaced with 150 µL 10% formalin solution. Immediately, 100 µL supernatant was removed and replaced with 100 µL
fresh 10% formalin solution. The plates were fixed for 12 min at RT in sterile conditions. To remove the formalin solution, 150 μL was aspirated and replaced with 150 μL sterile PBS. Immediately, a further 100 μL supernatant was removed and replaced with 100 μL fresh PBS containing 0.02% sodium azide. To prevent evaporation, a sealing film was placed over the plate prior to replacing the lid, and plates were stored at 4°C until use.

To prepare the plates for imaging, a scalpel was used to cut out sections of sealing film around the wells to be stained. Using a multichannel pipette, all PBS was removed from the wells required, by aspirating liquid from the edge of the wells without touching the cells. The cells were then permeabilised with 0.2% Triton X-100 in PBS by applying 100 μL down the side of the well. The plates were incubated at RT for 15 min and the blocking solution was prepared. To block non-specific binding of primary antibody to antigens, the cells were blocked with PBS containing 1% BSA and 10% gt or 10% dk serum depending on the species the secondary antibody was raised in. Once permeabilisation was complete the solution was removed from the side of the well, without touching cells. Blocking solution was applied at 100 μL per well and incubated for a minimum of 1 h at RT. Whilst blocking, the primary antibodies were prepared as follows (dilutions for each antibody used can be viewed in Table 4). For high content imaging (HCI), antibody combinations were accumulated and labelled as 'well sets'. The same well sets were applied to each clone for assessment and completed at the same time where possible, to avoid variability. All well sets used in this project are detailed in Table 5. Primary antibodies were diluted in PBS containing 1% BSA and 50 μL was applied per well to minimise antibody usage. To apply, the blocking solution was removed and primary antibody solution applied down the side of the well. Plates were incubated for either 2 h at RT or 4°C overnight with no agitation. Excess primary antibody was removed with a series of wash steps: 150 μL PBS was added directly to wells to take the volume to 200 μL. 150 μL supernatant was removed and replaced with a further 150 μL fresh PBS. Plates were then incubated for 5 min and the process was repeated a further three times. During the last wash, Alexa Fluor-conjugated secondary antibodies were prepared at a 1:1000 dilution in PBS and protected from light. All supernatant was removed from wells and 100 μL secondary antibody solution was applied down the side of wells. The plates were protected from light and incubated at RT for a maximum of 1 h. To wash the cells, 100 μL PBS was added directly to wells to take the volume to 200 μL. Washing steps were then completed as described above, twice. To stain the nuclei, a 2x Hoechst solution (2 μg/mL) was prepared. Half of the washing buffer (100 μL) was removed from wells and replaced with 100 μL 2x Hoechst staining solution. The plates were incubated in the dark for 5 min at RT prior to a final two rounds of washing as described above. The stained wells were then re-covered and plates were stored at 4°C protected from light until imaged. Immediately prior to imaging, 100 μL supernatant per well was removed. Plates were typically imaged immediately.
Table 5. Well sets used in this thesis. This table lists primary and corresponding secondary antibodies, optimal exposure time (ms) and Z plane (μm) at which all images were acquired using Opera Phenix HCI. [donkey - dk; goat - gt; mouse - m; rabbit - rb; rat - rt; chicken - ch]

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To measure immunofluorescence of stained wells, the Opera Phenix HCl platform was used (Perkin Elmer). This is a semi-automated imaging device that takes confocal images in a high throughput manner. Plates were inserted into the Phenix and for each Well set, an imaging setup was determined. To identify the optimal exposure time for each fluorophore, a series of snapshots were taken at increasing exposure times. The optimal exposure was determined as the time necessary to produce strong specific signal, but minimise background signal. To identify the optimal Z plane for each antibody, a Z stack was taken in test mode covering either 10 or 20 μm depending on cell density. Each fluorophore was assessed individually and the plane at which staining gave the optimal expected staining pattern was input into experimental set up. Finally, exposures were checked again to ensure that the Z plane had not altered the signal. Where possible, Z planes were kept constant within a Well set. For example, expected

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<td>Z (μm)</td>
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nuclear staining was imaged at the same Z as Hoechst, however in some cases it was not possible to keep this constant across the four wavelengths. The experimental set up was completed by selecting the desired wells to be imaged, a minimum of twenty fields of view (FOV) were selected randomly and acquired for each well resulting in a minimum of 120 FOV per clone, per Well set. Details of exposure times and Z planes for each experimental set up are documented in Table 5. The acquired images were automatically uploaded to Columbus (Harmony), the inbuilt analysis software, where analysis scripts were designed as described below.

2.5.2.3 Columbus script analysis
The Opera Phenix is supported by a cloud based analysis software called Columbus, which can be used to design image analysis scripts using in-built analysis parameters. For each Well set used in this project, a unique analysis script was designed, optimised for cell type, and run in batch mode across all plates to be analysed. An example of a Well set analysis script is shown in Figure 2. 4. Briefly, a representative image was selected from the 120 collected per clone. In the image analysis tab on Columbus, a script was constructed from building blocks provided by the software. These include, but are not limited to: ‘Find Nuclei’, ‘Find Cytoplasm’ and ‘Find Cells’. Each of the building blocks contained several methods of segmentation, which were manually applied to the image to find the most suitable method. The online help document was frequently referred to, as particular methods are associated with distinct cell-types; the ‘neuronal cell’ method was most frequently used. Within each method, the threshold and parameters could be further fine-tuned to provide the closest alignment with the staining pattern observed.

Each analysis script began with finding nuclei, which were then subject to morphological and intensity analysis. These parameters were used to identify pyknotic nuclei, signalling non-viability, and thresholds were established which enabled the selection of viable nuclei from the total population (40 μm < area > 200 μm; intensity < 1850). All subsequent measurements were calculated as a percentage of viable nuclei. Each fluorophore, corresponding to specific antibody staining, was assessed individually, with the primary aim of calculating the percentage of positive cells from the viable cell total. These data were then collated in the ‘Define Outputs’ tab, where the user could script formulas which would then produce the percentage of positively stained cells. The completed analysis file was saved to the Columbus data base. In order to avoid bias within an experiment, the same analysis script was applied to each clone stained with the corresponding Well set. To perform this, users navigated to the ‘Batch Analysis’ tab and selected the analysis script to be used. The user then selected all the data to be analysed with this script which was run by Columbus, and saved alongside the original imaging files. To access the data, the analysis file for each clone was downloaded and copied into NotePad, to provide a text version, before copying to Microsoft Excel. At this point, the average of each defined output was calculated, alongside the standard deviation. These values were then copied to GraphPad Prism for further analysis.
2.5.3 Super resolution microscopy (SIM)

Super resolution fluorescent microscopy was carried out in collaboration with Dr Edward J Smith (Bates laboratory) Institute of Neurology, UCL. Cells were prepared on 13 mm glass coverslips as previously described (2.5.1). Super resolution fluorescent images were captured using a Nikon Eclipse Ni-E N-SIM Super Resolution system and Andor Ixon camera at 100x magnification. Images were saved as ND2 files and processed using NIS Elements AR software with n-SIM module. Final images were exported as TIFF image files and AVI videos.

2.5.4 IncuCyte longitudinal imaging

A series of longitudinal imaging experiments were designed in order to assess morphological differences between genotypes in MSNs and CPNs in baseline conditions as well as after treatment (BDNF withdrawal, conditioned media experiments). PSC-derived neurons were seeded into PE plates at a density of $1.5 \times 10^4$ cells per well, with six wells per clone per condition. Seeding took place 24 h prior to the start of longitudinal imaging to allow the cells enough time to attach and recover from Accutase passage.
Plates were placed in an IncuCyte S3 live-cell imaging system (Essen Bioscience) which was maintained at 37°C [5% CO₂/O₂], and an initial image was taken after 30 min incubation to ensure any condensation build-up had evaporated and would not confound imaging. Plates underwent a half media change within 24-48 h post insertion, and this was timed to follow image procurement as closely as possible so that the cells had 6 h to recover prior to the next image acquisition. Images were acquired from four FOV per well, every 6 h for a minimum of 72 h. Phase contrast image sets were analysed using IncuCyte S3 2018B software (Essen Bioscience), and the inbuilt ‘NeuroTrack’ analysis was used to quantify neurite processes and cell bodies (Figure 2.5). Typical settings were: Segmentation Mode = Brightness; Segmentation Adjustment = 1.2 Background: Cells; Min Cell Width = 6 μm; Neurite Filtering = Best; Neurite sensitivity = 0.5; Neurite Width = 1 μm.

![Figure 2.5. Mask image of NeuroTrack software.](image)

The in-built NeuroTrack software segments the image FOV into either cell body clusters (blue) or neurites (yellow) for each image and time point. The software then automatically extracts the data e.g. average neurite length (mm). Image is the sample image provided by the EssenBioscience website.

### 2.5.5 Fluo-4 live cell imaging

To investigate the presence of calcium transients within cultured neurons, the calcium binding dye Fluo-4 was used. Labelled calcium indicators are molecules that exhibit an increase in fluorescence upon binding Ca²⁺. Fluo-4, is commonly used as the non-fluorescent acetoxymethyl ester (Fluo-4 AM) which is cell permeant and cleaved inside the cell to give the free, fluorescent Fluo-4 which is well-excited by the 488 nm line of the argon-ion laser. By conducting time-series recordings on a confocal microscope it was possible to visualise the fluctuations of Ca²⁺ within the cell. Calcium transients are indicative of action
potential ability as they require the functionality of ion channels within the cell membrane, required for action potential propagation along the axon. Mature MSN cultures at various time points (range d40-d135) were washed with HEPES buffer [115 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 2.4 mM K₂HPO₄ diluted in sterile water, pH 7.4 adjusted by HCl] to remove cell debris prior to incubation with 200 mM Fluo-4 diluted in HEPES buffer for 30 min at 37°C. Cultures were protected from light and cells were imaged using repeated time-series recordings taken by confocal microscopy for a maximum of 20 min post-incubation.

2.5.6 GFP transient transfection
A transient transfection of PSC-derived neurons was designed and optimised using Lipofectamine 2000 (ThermoFisher), Opti-MEM (Gibco) buffer and pmaxGFP DNA. A range of plasmid concentrations were trialled and assessed for transfection efficiency, expression and signal strength (0.31 μg, 0.47 μg, 0.625 μg and 1.25 μg pmaxGFP vector [stock = 1 μg/μL in 10mM Tris pH 8.0, Lonza]). The optimal signal expression for imaging was deemed to be between 0.625 and 1.25 μg, therefore subsequent transfections were carried out at 1 μg.

Briefly, the number of reactions required was calculated for the experiment and solutions made up in 7 mL bijou tubes (Thermo) as follows: 25 μL Opti-MEM + 2 μL Lipofectamine 2000; 62.5 μL Opti-MEM + 1 μL (1 μg) pmaxGFP (Lonza). Solutions were triturated thoroughly prior to adding 27 μL GFP solution to Lipofectamine solution (1:1 volume ratio). Lipofectamine + GFP solution were triturated ten times and incubated at RT for 5 min. For each well to be transfected, 10 μL of Lipofectamine + GFP was added dropwise to culture wells and incubated at 37°C for 48 h. Expression of GFP increased over time in culture and was maintained with minimal toxicity for 20 days.

2.6 Cell-based assays

2.6.1 Lactate dehydrogenase (LDH) cytotoxicity assay
CytoTox 96 Non-Radioactive Cytotoxicity Assay kits (Promega) were used to quantitatively measure lactate dehydrogenase (LDH) levels in cell culture supernatants. LDH is a cytosolic enzyme that is released from cells undergoing apoptosis or necrosis, therefore this kit broadly measures levels of cell death within a culture. The assay was conducted as per the manufacturer’s instructions. Briefly, to assess levels of LDH in cell culture medium, 50 μL cell supernatant was transferred to a sterile 96-well plate (Nunc), leaving 50 μL culture media on the cells. To induce cell lysis, enabling quantification of total cell death, 5 μL of 10X Total Lysis solution (supplied) was added to cells, and incubated for 45 min at 37°C. LDH assay powder was dissolved in 12 mL assay buffer and protected from light. The reconstituted assay solution was added at 50 μL per well to both supernatant and total cell death samples and incubated at RT for 30 min protected from light. Stop solution (supplied) was added to plates prior to reading on a Tecan Spark spectrophotometer at 450nm and data was analysed using Microsoft Excel and GraphPad Prism. The percentage cell death of each sample was calculated using the following equation:
2.6.2 Bicinchoninic acid (BCA) protein assay

The Pierce BCA protein assay (Thermo Fisher) was used to determine total protein concentrations from samples. Briefly, a serum albumin protein standard was generated using serial dilutions as directed per the manufacturer's instructions. Cell pellets were removed from -80°C and reconstituted in RIPA buffer supplemented with 1x protease inhibitor and 1x phosphatase inhibitor. Pellets were homogenised using vigorous trituration (>10 passes) to produce a smooth cell lysate. For aged neurons, which have a higher lipid content due to extensive neuronal processes and subsequent increased plasma membrane volume, it was sometimes necessary to homogenise the cell lysate further by passing through sterile needles of increasing gauge (19G - 23G). Once fully homogenised, 10 μL of sample was added to wells of a 96-well plate, alongside protein standard samples. The working reagent (WR) of BCA buffer was generated by mixing 50 parts buffer A, with 1 part buffer B, and 200 μL WR was added to each standard/sample. The plate was protected from light and incubated at 37°C for 30 min. After cooling to RT, the plate was read on a Tecan Spark spectrophotometer and absorbance values were measured at 562 nm. To calculate protein concentrations, a line of best fit was applied to the protein standard curve. The raw value for the 'blank' was subtracted from all experimental samples, prior to transposing raw sample values using the equation of the line.

2.6.3 Adhesion assay

To optimise the conditions for future adhesion assays, a pilot experiment was completed on PSCs. PSCs from a single well of a confluent 12-well plate were collected using Accutase. Cell pellets were re-suspended in 5 mL E8+ and 50 μL cell suspension was applied to a Geltrex coated 24-well plate, containing 300 μL E8+ per well. Cells were added in reverse order instalsments of 0, 5, 10, 15, 30 and 45 min at 37°C. At the end of the last time-point, the plate was removed from the incubator and shaken vigorously to detach loosely adhered cells, leaving only those which had formed a strong attachment. Wells were then washed vigorously twice with PBS to further remove loosely attached cells. The remaining cells were counted manually using 10x magnification on an AxioVert.A1 inverted microscope (Zeiss) and images were captured by an AxioCam ICam1 camera (Zeiss). Three FOV were acquired for each condition which was run in duplicate. This pilot experiment indicated that 15 min attachment was optimal, as a moderate percentage of cells could form attachments, but the cultures remained sparse enough to enable visual quantification. These conditions were then used for all subsequent adhesion experiments.

2.6.4 BDNF withdrawal

The adapted Arber et al., (2015) protocol used to generate MSNs was further adapted to allow for assessment of acute BDNF withdrawal, and BDNF deficiency on MSN viability. For acute assessment, d36 MSNs were subjected to BDNF withdrawal for a period of either 24 or 48 h. To deplete existing BDNF from culture media, an 80% volume media change was completed with

\[
\text{Percent cytotoxicity} = 100 \times \frac{\text{Experimental LDH Release}}{\text{Total LDH Release}}
\]
MSN post-26 media supplemented with activin A and GDNF at d36. This volume ensured that the meniscus remained intact above cells to prevent sheathing, but reduced the concentration of BDNF in culture media to less than 2 pg/mL. Parallel cultures underwent the same process with fully supplemented MSN post-26 media. Cells assayed for 48 h BDNF withdrawal completed this process again after 24 h. The half-life of LDH in culture media is nine hours, therefore to ensure optimal accumulation of LDH and thus accurate measurement of cell death, a half media change was completed 5 h prior to assay termination. After 24 or 48 h BDNF withdrawal, cells and samples were subject to LDH assay as described (section 2.6.1).

Chronic BDNF deficiency was carried out as follows: during the second passage, MSNs were seeded into two culture plates per experiment in identical conditions (number of wells, density, volume). At d26, one plate underwent a full media change into fully supplemented MSN post-26 media. The second plate was subject to a full media change into MSN post-26 media supplemented only with activin A and GDNF. The cells were cultured in these conditions, undergoing half media changes every 48 - 72 h until d50 in culture.

2.6.5 Potassium chloride and glutamate stimulation
Cortical neurons were seeded in 24-well plates at a density of 5 x 10^4 cells per well. At d50, 90% of culture media was removed from wells and replaced with either control media [N2B27], or media supplemented with 50 μM potassium chloride (KCl) (Sigma), or 200 μM glutamate (Sigma). Cells were incubated at 37˚C for 30 min. The conditioned media from cells, and cell lysates were collected using Accutase. Samples were either used immediately, or snap-frozen on dry ice prior to being run on a BDNF ELISA as described below.

2.6.6 Enzyme-linked immunosorbance assay
All BDNF ELISAs (Sigma) were carried out as per the manufacturer's instructions. Briefly, all reagents and samples were equilibrated to RT; all subsequent steps are carried out at RT unless otherwise stated. The protein standard curve was generated by performing serial dilutions of reconstituted BDNF protein standard (supplied), providing a range of 16 - 0.066 ng/mL plus a blank control (Assay Buffer B, supplied). Standards and samples were added to the ELISA plate and allowed to incubate under 500 rpm agitation for 3 h, or overnight at 4˚C. Conditioned media samples ran undiluted, cell lysate samples were diluted 5-fold in Assay Buffer B (supplied). The plate was subject to 4x 200 μL/well washes using 1x ELISA wash buffer (supplied), prior to primary antibody addition at a dilution of 1:80 in Assay Buffer B. Primary antibody was incubated under agitation for 1 h prior to washes as previously described. Streptavidin-HRP (supplied) was then added to the plate at a 1:200 dilution in Assay Buffer B, and incubated for 45 min under agitation. The plate was washed again as previously described and vigorously tapped against paper towels to ensure all moisture was removed. Finally, ELISA colorimetric TMB reagent was added to the plate and incubated under agitation for 30 min protected from light. Stop solution was added (50 μL) and the plate was read at 450 nm on a Tecan Spark spectrophotometer.
The blank standard value was subtracted from sample absorbance values, prior to deriving the protein concentration of samples using the standard curve. BDNF protein concentrations were normalised to total protein concentrations of corresponding cell lysate samples as assayed by BCA (Section 2.6.2).

2.7 Microfluidic chamber assembly

Microfluidic chambers (MFC) are devices that can be engineered to fluidically isolate two or more distinct chambers. They were used in this project to replicate the physical separation of CPNs and MSNs that is normally found in the brain, in an attempt to recapitulate the CS pathway as optimally as possible. All microfluidic work was conducted in collaboration with Prof Giampietro Schiavo. The MFC insert template was previously designed and optimised by members of the Schiavo laboratory.

2.7.1 Production of MFC polydimethylsiloxane (PDMS) insert

The substrate to make the MFC gel inserts was made as follows: polydimethylsiloxane PDMS solution (VWR) was weighed out to account for 4 g/MFC, and curing agent added to a 10% w/v final concentration. The reagents were mixed thoroughly for 5 min before being placed in a vacuum chamber for a minimum of 1 h. To remove debris and dust from MFC templates, three rounds of cleaning took place using clear Scotch tape. Once prepared, the PDMS solution was poured into each MFC template, any bubbles formed were removed from the chambers and micro-channel areas using a P200 pipette tip. The MFCs were then baked in an electric oven at 67°C for a minimum of 1 h. Once baked, the MFCs were allowed to cool for 10 min, before the hardened gel MFC inserts were removed from the templates using sculpted tweezers. Excess gel was removed and wells were generated by cutting over the corners of each of the chambers to create entrance/exit routes for media/cells.

2.7.2 Decontamination and plate preparation

To bond the MFC gel insert to tissue culture dishes, two methods were initially used depending on the length of experiment. These included plasma bonding, which also sterilised gel inserts, and reversible PDL bonding (Park et al., 2006). During the course of this project, the plasma cleaner became unreliable therefore all data presented was produced using the reversible PDL bonding method. As this method does not include a decontamination or sterilisation step, a protocol was developed to encourage sterile conditions. Once gel inserts had been cut to size and access provided to the chambers, the inserts were soaked in 75% ethanol under agitation for a minimum of 4 h. The inserts were then removed and allowed to air dry in a laminar flow cabinet to enable the ethanol to evaporate. Inserts were then submerged in sterile distilled water to remove any traces of residual ethanol before air drying. During this time, 35 mm glass bottom dishes were coated with 0.1% PDL in water, and incubated at 37°C for a minimum of 4 h. Dishes were then washed three times in distilled water and allowed to air dry in a laminar flow cabinet.
2.7.3 MFC assembly

Once both the gel insert and glass bottom dish were sterilised and coated, the gel insert was dropped from a height (approximately 10 cm) using sterilised tweezers into the centre of the dish. This process ensured that a tight seal was made between insert and coating substrate, and negated the need to form a seal by applying additional pressure - this distorts the morphology and dimensions of the micro-channels. Immediately after bonding, the MFCs were primed with 1% BSA prior to coating with 20 ng/mL laminin for a minimum of 2 h at 37˚C. Laminin solution was washed out with two culture media washes prior to cell seeding.

2.7.4 Cell seeding of MFCs and maintenance

Several rounds of optimisation were completed with regard to seeding density. Briefly, CPNs were harvested at d49 by Accutase. Cell density was calculated to account for 4 x 10⁴ cells per MFC for axonal crossing studies described below, or 5 x 10⁴ cells per MFC for co-culture experiments. The protocol to seed cells into the MFC can be observed in Figure 2. 6. The cell suspension was then centrifuged at 100xg for 4 min to pellet the cells, which were then re-suspended in N2B27 medium at a volume of 5 μL per MFC (Figure 2. 6A). The recipient MFC had N2B27 media removed from the wells and was laid horizontally. Using a 10 μL pipette, 5 μL of cell suspension was injected into the somal chamber by inserting the pipette tip directly into the chamber entrance. To introduce hydrostatic pressure to the device and prevent somal chamber flow-through, 10 μL N2B27 medium was then applied to both the entrance and exit of the axonal chamber (Figure 2. 6B). MFCs were left to incubate at 37˚C and maintained in a horizontal position for a minimum of 30 min. Finally 150 μL, and 100 μL culture media was added drop-wise to the somal and axonal chamber wells, respectively (Figure 2. 6C), a process repeated after 24 h incubation. Cultures were maintained with half media changes every 48 h. This process involved removing 100 μL from each well of the MFC and replacing with fresh N2B27 media.
2.8 Use of MFCs to assess axons

2.8.1 Cell seeding

For axonal crossing experiments, CPNs were seeded as described above at a density of $4 \times 10^4$ cells per somal chamber. A total of three MFCs were used per clone, and minimum of two clones of each line were used per experiment. Multiple experiments were performed which incorporated multiple differentiations, therefore the analysis accounts for variability in differentiation efficiency, MFC bonding and culture conditions.

2.8.2 Axon crossing

2.8.2.1 Pilot experiment

After 24 h incubation post-seeding of clones 22Q (1/2) and 75Q (1), MFCs were placed in a plate holder and observed under 20x magnification on an AxioVert.A1 inverted light microscope (Zeiss). The pilot experiment consisted of four measures as depicted in Table 6. Measure 1 investigated the ability of CPNs to project their neural processes into micro-channel entrances, and was measured every 48 h. Measure 2 assessed the frequency of neural processes successfully crossing through micro-channels and emerging into the axonal chamber; measure 2 was assessed every 24 h. Measure 3 was completed on the final day of experimentation, and assessed the efficiency of neural projection by dividing measure 2 by measure 1. Finally, measure 4 assessed the formation of neural networks post-projection into the axonal chamber in terms of morphology.
Table 6. Description of measures assessed in pilot axon crossing experiments.

<table>
<thead>
<tr>
<th>Biological process</th>
<th>Measure</th>
<th>Description</th>
<th>Days assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>How exploratory are the axons?</td>
<td>1. Channel occupancy</td>
<td>The number of micro-channels (total 68/MFC) occupied by 1 or more neural projection.</td>
<td>1, 3, 5, 7</td>
</tr>
<tr>
<td>How fast are the axon cytoskeletal dynamics?</td>
<td>2. Latency to cross</td>
<td>The number of axons successfully projected through to the axonal compartment.</td>
<td>Every 24 h</td>
</tr>
<tr>
<td>How efficient is the culture in terms of axon projection?</td>
<td>3. Projection efficiency</td>
<td>The total number of axons projected through to the axonal chamber divided by the total number entered.</td>
<td>End of experiment (d7)</td>
</tr>
<tr>
<td>Are the neurons capable of generating an axonal network?</td>
<td>4. Network complexity</td>
<td>An assessment of how complex the projected axonal networks are: axonal interactions and branching points, evidence of synaptic swellings. Scoring system as follows: 0 = no networks formed/no interactions. 1 = axons are in close proximity to each other. 2 = axons look to be interacting with each other in a simple network. 3 = axons are interacting with each other in a complex network - multiple interaction points on same axons and multiple axon interactions.</td>
<td>End of experiment (d7)</td>
</tr>
</tbody>
</table>

2.8.2.2 Full experiment

The data collected in the pilot experiment led to identifying the optimal conditions required for axonal crossing. Furthermore, the number of measures was reduced to focus on measure 2 - the frequency and latency of axonal crossing (Figure 2.7). Briefly, every 24 h, MFCs were observed under 40x magnification on an AxioVert.A1 (Zeiss) microscope. Using a manual cell counter, the number of neural processes emerging into the axonal chamber from micro-channels was counted. In conditions where multiple processes emerged from the same micro-channel, every effort was made to accurately count the number of individual processes. In some conditions however, the resolution of the microscope limited this, therefore a weighted system was used to quantify axons: where processes were sparse they scored 10; half-filled the micro-channel, 20; and filled the micro-channel scored 40. The tally of projected processes was documented for each MFC every day for seven days and recorded in Microsoft Excel.
Figure 2. 7. Diagram of axon crossing full experiment. A) CPNs were seeded as previously described and allowed to adhere and begin projecting for one day. B) For a total of seven days, neuronal processes were counted every 24 h as they emerged into the axonal chamber (right panel) to give a longitudinal assessment of axonal projection frequency and latency.

2.9 Electrophysiological recordings

All electrophysiological work was conducted in collaboration with Yichen Qui of Dr Stephanie Schorge's laboratory (UCL's Institute of Neurology). To prepare the cultures for proposed experiments, MSNs were seeded at a density of 5 × 10⁴ for patch clamp recordings. Cultures were maintained as previously described, with half media changes performed every 48 h. Patch clamp recordings were performed on cultures matured to d60 and were completed within seven days. Recordings from a minimum of nine cells per condition was required to enable reliable conclusions from data. Patch clamp conditions were replicated as described in Arber et al., (2015). Briefly, for current-clamp recordings of cultured neurons, the internal solution contained (in mM): 126 K-gluconate, 4 NaCl, 1 MgSO₄, 0.02 CaCl₂, 0.1 BAPTA, 15 Glucose, 5 HEPES, 3 ATP-Na₂, 0.1 GTP-Na, pH 7.3. The extracellular (bath) solution contained (in mM): 2 CaCl₂, 140 NaCl, 1 MgCl₂, 10 HEPES, 4 KCl, 10 glucose, pH 7.3.

Neurons with unstable resting potential and/or holding current > 200 pA were discarded. Bridge balance compensation was applied and the resting membrane potential was held at -70 mV. A current step protocol was used to evoke action potentials (APs), by injecting 500 ms long depolarizing current steps of increasing amplitude from -20 pA (Δ 10 pA). Recordings were acquired with a Multiclamp 700A amplifier (Axon Instruments, Molecular Devices) and a Power3 1401 (CED) interface combined with Signal software (CED), filtered at 10 kHz and digitized at 50 kHz. Action potentials were selected only if the voltage crossed 0 mV.

2.10 Corticostriatal co-culture

2.10.1 Conditioned media experiments

Conditioned media was collected from 90% confluent CPNs plated in 12-well plates at d50. Briefly, 24 h prior to collection, CPNs underwent an 80% media change with fresh N2B27. Twenty-four hours later, the media was collected from multiple wells and pooled prior to
centrifugation to remove cellular debris. The supernatant was then collected, aliquoted and
snap frozen for use in future experiments.

2.10.1.1 CPN conditioned media onto MSNs

At d30, MSNs were passaged using Accutase into PE plates at 2 x 10⁴ cells per well. Three
control clones and three HD clones were plated in the inner sixty wells, thus each clone
occupied ten wells. At d36, MSNs received a 90% media change to remove as much MSN post-
26 media as possible. CPN conditioned media was thawed on ice and allowed to reach RT.
Each well containing MSNs received 100 μL CPN conditioned media, MSN control (MSN post-
26 media fully supplemented) or cortical control (N2B27) in the plate layout as depicted below
(Table 7). The plate was incubated at 37˚C for a minimum of 2 h prior to transfer to the
IncuCyte. Plates underwent a half media change of conditioned media after 48 h and the
experiment was terminated 48 h later (total conditioning time 96 h). The media was collected
and subject to LDH assay, however cells were not lysed to provide a value for total cell death.
Instead, cells were fixed with 10% formalin solution as previously described, and subject to HCl
immunofluorescence; the total cell count was used to normalise LDH absorbance values as
described in 2.6.1.

Table 7. Plate layout for CPN conditioned media experiment on d36 MSNs. Each condition was run
in duplicate per MSN clone. This schematically shows the plate layout of the inner 60 wells of the PE
plates seeded with MSNs derived from cultures of 22Q, 30Q and 75Q clones. Each MSN clone was
seeded into a row of 10 wells, with 2 wells per treatment. MSN control = supplemented MSN post-26
media; CPN control = N2B27 media.

<table>
<thead>
<tr>
<th>MSN clone</th>
<th>Conditioned media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSN control</td>
</tr>
<tr>
<td>22Q (1)</td>
<td></td>
</tr>
<tr>
<td>22Q (2)</td>
<td></td>
</tr>
<tr>
<td>30Q</td>
<td></td>
</tr>
<tr>
<td>75Q (1)</td>
<td></td>
</tr>
<tr>
<td>75Q (2)</td>
<td></td>
</tr>
<tr>
<td>75Q (3)</td>
<td></td>
</tr>
</tbody>
</table>

2.10.2 MFC co-culture cell seeding

To construct physical CS co-cultures, PSC-derived CPNs and MSNs were seeded into somal
and axonal chambers of an MFC respectively (Figure 2. 8). The seeding occurred
simultaneously and followed the same protocol as described in 2.8.4. Briefly, 5 x 10⁴ CPNs were
injected into the somal chamber, followed by 4 x 10⁴ MSNs injected into the axonal chamber.
Each cell type was maintained in its own media. A small volume increase was introduced to the
CPN chamber which maintained the positive pressure towards the MSN chamber, whilst also discouraging MSN retrograde projection.

Figure 2.8. Corticostriatal co-cultures in MFCs. To generate CS co-cultures in MFCs, cortical and MSN cell suspension were seeded into somal and axonal compartments at $5 \times 10^4$ and $4 \times 10^4$ respectively. The cell suspension was injected into top chamber entrances in 5μL volume and allowed to adhere for 30 min. Subsequently, 150μL and 125μL N2B27 media and supplemented MSN post-26 media was added drop-wise to the CPN and MSN chambers respectively. Co-cultures were maintained with twice weekly media changes in which 100μL supernatant was removed from each well and replaced with fresh media for the corresponding cell type.

2.10.3 Co-culture maintenance

Co-cultures were maintained with twice weekly media changes. Briefly, 100 μL supernatant was removed from each of the wells, and replaced with 100 μL fresh N2B27 media (cortical wells) or fully supplemented MSN post-26 media (MSN wells).

2.10.4 Co-culture assessment

2.10.4.1 Pilot experiment

As no previous reports of human PSC-derived co-cultures successfully generated within MFCs were found in the literature, a pilot experiment was designed in order to discover the optimal conditions and timeline required for assessment. Due to cell genotype availability, the pilot experiment was completed with 75Q CPNs, projecting onto 22Q MSNs (Table 8). A total of three MFC co-cultures were produced for each genotype combination, so that a semi-longitudinal assessment could be completed. The initial experimental design called for levels of cell death to be assessed after 10 days co-culture, therefore one MFC from each genotype combination was fixed as previously described and subject to activated caspase-3 staining. The remaining two MFCs per combination were maintained in culture. At d22 co-culture, a second
MFC was fixed and subject to immunofluorescence labelling of synaptic markers. The final MFC was monitored closely to identify the maximum length of time the co-cultures could survive in the MFCs without compromising cell viability; these cultures were terminated at d30 co-culture (4 weeks). The results from these pilot experiments contributed to the design of the full experiment detailed below.

### 2.10.4.2 MFC based CS co-cultures

Due to the success of the pilot experiment, a 4 x 4 experiment was designed whereby four clones (two x 22Q, two x 75Q) were selected in both CPN and MSN format. At d40, both CPNs and MSNs were harvested using the Accutase passage protocol, and seeded into somal and axonal compartments of an MFC, respectively. The somal chamber received $5 \times 10^4$ CPNs whilst $4 \times 10^4$ MSNs were seeded into the axonal chamber. For each co-culture combination as depicted in Table 8, two MFCs were generated. A total of sixteen co-culture combinations were constructed, resulting in 32 MFCs for assessment in total. After 24 h incubation, images were taken of each co-culture MFC on an AxioVert.A1 microscope using an AxioCam 503 mono (Zeiss), under 20x magnification. Further images were acquired after 21 days, and 30 days co-culture respectively. MFCs underwent half media changes every 72 h and were monitored closely every 24 h for signs of cell death or deteriorating conditions.

**Table 8. Experimental design of co-culture pilot experiment, and full cohort assessment.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Somal chamber CPN clone</th>
<th>Axonal chamber MSN clone</th>
<th>Co-culture genotype</th>
<th># MFCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pilot</strong></td>
<td>75Q (1)</td>
<td>22Q (1)</td>
<td>75Q/22Q</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>75Q (2)</td>
<td>22Q (1)</td>
<td>75Q/22Q</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>22Q (2)</td>
<td>22Q (1)</td>
<td>22Q/22Q</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>22Q (2)</td>
<td>75Q (1)</td>
<td>22Q/75Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>22Q (3)</td>
<td>22Q (1)</td>
<td>22Q/22Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>22Q (3)</td>
<td>75Q (1)</td>
<td>22Q/75Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>75Q (2)</td>
<td>22Q (1)</td>
<td>75Q/22Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>75Q (2)</td>
<td>75Q (1)</td>
<td>75Q/75Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>75Q (3)</td>
<td>75Q (1)</td>
<td>75Q/22Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>75Q (3)</td>
<td>75Q (1)</td>
<td>75Q/75Q</td>
<td>2</td>
</tr>
</tbody>
</table>
2.10.4.3 Co-culture LDH

To assess the levels of cell death in MFC co-cultures longitudinally, repeated media samples were taken to run on LDH assay. After seven days co-culture, MFCs were tilted 45° and 250 μL was removed from both of the bottom wells. After approximately 30 seconds, two samples of 50 μL were collected which contained the ‘flow through’ media from the cells contained within the chamber. These samples were placed in individual wells of a 96-well plate for LDH assessment. The top chambers received 100 μL fresh culture media which was allowed to flow through due to gravity.

After 22 days co-culture, one of the MFCs for each co-culture combination was kept horizontal and 2x 50 μL samples were taken from the top cortical, bottom cortical, top MSN and bottom MSN wells, respectively. MFCs were then tilted to 45° to allow the flow through from the cell chambers to accumulate in the bottom well. Subsequently 2x 50 μL samples were taken and labelled as flow through #1 and #2 respectively. The MFCs were then devoid of media, and were fixed with 10% formalin by applying 200 μL to both of the top wells and allowing it to flow through for 15 min at RT. The formalin was removed and MFCs were subject to 3x PBS washes prior to further experimentation. The collected samples were subject to LDH assay as previously described (2.6.1). In order to control for cell density, nuclear counts were completed of both cortical and MSN chambers for each co-culture. Each image taken, represented 6/68 micro-channels in height, and 1/5 of the chamber width. To estimate the total cell number in each chamber, the nuclear count value was multiplied by 11.33 (68/6) and further multiplied by 5. LDH raw values had background intensity removed prior to normalisation by the corresponding chamber cell number. This process was repeated with the remaining MFC for each co-culture combination after 30 days co-culture.

2.11 Statistical analysis

Due to the complexity surrounding biological replicates in PSC investigations (as for each line, all material originates from the same donor), every effort was made to produce sufficient replications of each experiment to account for both biological and technical variation. To account for the potential variability introduced as a result of Sendai reprogramming, experiments were completed in a minimum of two clones per genotype i.e. a figure stating a result for 22Q is the combined average of measurements made from at least two 22Q clones (1/2/3). To overcome variability introduced by the differentiation process, experimental results from multiple differentiations were pooled for analysis. Finally, all experimental conditions (unless otherwise stated) were run with at least two internal replicates per condition. For experiments where culture variation was expected (e.g. due to possible variability introduced during MFC construction) a minimum of 3 independent culture plates were assessed. To facilitate the interpretation of the results presented within this thesis, and aid the reader in understanding the sample size from which results were derived, the following formatting code can be used:
N = biological replicates per genotype (individual donors)
n = experimental replicates per genotype (number of clones)
\( n \) = technical replicates per experiment

Unless otherwise stated, data were processed initially in Microsoft Excel, prior to transfer to GraphPad Prism v8.0 for Windows. Here, all graphs were generated and statistical analysis completed. Figures were designed and formatted in Adobe Illustrator. Data are presented as mean ± standard deviation (SD), or in some cases as mean ± standard error of the mean (SEM). Statistical \( p \)-values are graphically presented as follows: * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \), **** \( p<0.0001 \).

To compare the mean difference between two groups, such as MSN cell viability at baseline in the HD family series, a two-tailed Student's \( t \)-test was employed. To compare the mean differences between three or more groups during a single time point, e.g. MSN viability at baseline in the IsoHD series, a one-way analysis of variance (ANOVA) was used. When multiple independent variables were selected for an experiment e.g. IsoHD MSN viability assessment when treated with/without BDNF, a two-way ANOVA was used. In each case, where multiple comparisons were being calculated, Bonferroni or Tukey post-hoc testing was applied, so \( p \) values could be adjusted appropriately for multiple comparisons. To assess the mean differences occurring over time, where some data points may be missing e.g. CPN axon crossing experiments, a mixed effects analysis was employed, and multiple comparisons corrected for by Tukey post-hoc test. Repeated measures ANOVA was employed when assessing mean differences occurring over time when no data was missing (IncuCyte data), as well as linear regression analysis where appropriate.
Chapter 3 - Optimisation, characterisation and Huntington's disease phenotypes in PSC-derived MSN-containing cultures

3.1 Background

The pathology caused by mHTT is widespread across the CNS, however several cell types are selectively vulnerable and succumb to dysfunction and death early in the disease. The MSNs of the striatum are particularly vulnerable in HD; it has been well established for many years that MSNs are one of the principal cell-types lost in the disease (Bates et al., 2015). MSNs receive the majority of their excitatory innervation and trophic support from layer V CPNs via the CS pathway and together they assist in controlling coordinated movement (Bates et al., 2015; Cepeda et al., 2007; Ehrlich, 2012). The pathology exhibited by MSNs in HD and its models are substantial; the neurons exhibit an extensive range of different pathologies, some of which are dependent on the model system and organism in which investigation takes place. Human post-mortem studies have consistently demonstrated almost complete atrophy of the caudate and putamen regions in end-stage HD patients (Tippett et al., 2007; Vonsattel et al., 2011). Similarly, imaging data shows a gradual loss of neostriatal volume in correlation with disease progression, with evidence of atrophy more than ten years before onset (Georgiou-Karistianis et al., 2013; Wilkes et al., 2019). These data support the hypothesis that striatal degeneration and atrophy occurs prior to symptom onset.

Due to the difficulty in obtaining striatal tissue from pre-manifest or early-stage HD gene carriers, there is a lack of data regarding the effect of mHTT on human MSN biology, including how it contributes to dysfunction and pathology, especially in early stages of the disease. Therefore, most information in the field regarding HD cellular pathology in MSNs is derived from animal models, but also, more recently, from PSC-derived MSN models of HD.

3.1.1 MSN dysfunction in animal models of HD

As aforementioned, there are three main types of HD animal model widely used in the field. Knock-in, fragment and full-length models have all contributed to our understanding of MSN pathology in HD, but they all have limitations (reviewed in Raymond et al., 2011). Whilst fragment models such as the R6/2 display a very aggressive form of the disease, with mortality usually before 16 weeks of age, full-length models have a much longer, and some would argue more representative, disease progression typically developing over the course of two years; both types of model display the hallmark striatal and cortical atrophy observed in HD. Studies conducted in each of these models have identified electrophysiological and synaptic alterations of mHTT-expressing MSNs, including reduced synaptic and elevated extra-synaptic NMDA receptor currents that contribute to increased apoptosis (Milnerwood et al., 2012), a hyperpolarised resting membrane potential facilitating hyper-excitability (Ehrlich, 2012), and decreased dendritic spine density (Reis et al., 2011).

3.1.2 MSN dysfunction and pathology in PSC-derived models of HD

As human MSNs cannot be easily studied within HD patients, researchers have developed methods of studying human MSN biology by generating them either from PSCs or even directly
from fibroblasts, so that disease pathology can be studied *in vitro* (Takahashi and Yamanaka, 2006; Tousley and Kegel-Gleason, 2016; Victor et al., 2018). Due to the importance of striatal pathology in HD, there have been several publications describing the emergence of HD-associated phenotypes in PSC-derived or directly differentiated MSNs. In their 2012 paper, the HD iPSC Consortium completed an in-depth transcriptional analysis of PSC-derived MSNs expressing 21-33 CAG-repeats (controls) and 60, 109 and 180 CAG-repeats. The authors identified 1601 genes that were differentially expressed (DEG) in HD lines and the genotypes clustered separately during hierarchical clustering analysis (The HD iPSC Consortium, 2012). The same group went on to show that 29% of DEGs were attributable to developmentally associated genes, as well as axon guidance and growth cone functionality (HD iPSC Consortium, 2017). Two independent studies also identified a down-regulation of TrkB expression, the BDNF receptor expressed by MSNs, and up-regulation of the NMDA receptor subunit NR2B (Mattis et al., 2015; The HD iPSC Consortium, 2012). Combined, these two altered expression levels could be responsible for the enhanced levels of cell death observed in mHTT expressing lines. Indeed, decreased viability after BDNF withdrawal is a common finding in PSC-derived MSN studies (The HD iPSC Consortium, 2012; Xu et al., 2017b), but whether this cell-type is preferentially lost, or most susceptible, in such cultures is undefined (Mattis et al., 2015).

Most studies to date have not found evidence of mHTT-positive aggregate formation within cultured human neurons, however there have been two reports to the contrary. Nekrasov *et al* found that when cultured for six months, EM48+ HTT aggregates could be observed in the nuclei of directly-converted HD lines, a process which could be accelerated with the treatment of the proteosome inhibitor MG132 (Nekrasov et al., 2016). Directly-converted MSNs also present HD-associated phenotypes much more rapidly in culture, without the apparent need for ageing or exogenous stress (Victor et al., 2014). It is hypothesised that the directed differentiation process leaves epigenetic signatures intact within the cells (unlike somatic cell reprogramming techniques), therefore the differentiated cells retain the age-associated genomic modifications of the donor.

One important aspect of PSC-derived HD studies that is relatively under investigated to date, is the impact of the genetic background on cell line phenotypic presentation in culture. In 2017, Xu *et al*, observed a range of HD-associated phenotypes in a cellular cohort derived from non-related individuals expressing 33 and 180 CAG-repeats, as well as corresponding isogenic clones that had been corrected to non-pathogenic range from the 180 CAG line. It should be noted that although the cells were directed toward GABAergic fate, they were not explicitly characterised for MSN identity, instead termed forebrain neural cells. Transcriptional analysis identified 159 and 131 DEGs between non-isogenic control and HD PSCs and NPCs respectively, however, more than 50% of those DEGs were not reproduced when comparing between 180Q and isogenic controls. This suggests that the genetic background and associated variability of cells had as much of an impact on cell function as the HD mutation itself. Indeed, most experiments described above were also carried out using only a single clone per genotype; the Xu *et al* study highlighted the importance of using multiple clones during
experimentation to account for variability introduced by the reprogramming or genetic correction process.

The data produced from direct reprogramming, as well as PSC-derived neuronal studies, has contributed to our understand of human pathology in HD and the way it manifests. Although there are juvenile cases of the disease, HD is classically defined as an adult-onset disease, with symptom manifestation typically emerging in the fourth decade of life (Bates et al., 2015; Myers, 2004; Zuccato and Cattaneo, 2014). Animal models and human imaging data have shown that pathology begins well in advance of symptom emergence, but it has been questioned as to whether this would translate to human cellular models that are, in effect, more representative of the developing brain (Mehta et al., 2018). The studies discussed here have demonstrated that HD-associated phenotypes are occurring in the earliest stages of neuronal differentiation and persist through ageing in culture. This project wanted to further investigate these phenomena in a robust and reliable manner, as well as model environments such a chronic BDNF deficiency, which may be occurring throughout development and persist during ageing in the HD brain. To improve the reliability of data, this project was designed in such a way that multiple cell sources, genotypes and clones would be tested in order to control for phenotypic variability due to factors other than HTT polyQ-length.

3.2 Aims

1. Adapt the Arber et al protocol for PSC differentiation into MSNs to enable:
   - synchronisation of multiple differentiating lines
   - HCI experimentation and analysis
   - long term culture to assess age-related phenotypes
2. Characterise the full cohort of PSC-derived MSNs with a range of CAG repeat lengths, in terms of culture composition.
3. Determine whether MSN cultures from PSC lines with CAG lengths > 39 show reduced cell viability either basally or in response to stress.
4. Evaluate whether MSNs with HD-associated CAG lengths demonstrate additional or novel phenotypes in vitro over time.

3.3 Methods

A summary of methods used in this Chapter are outlined below. Full details of experimental protocols can be found in Chapter 2 - Material and methods.

Pluripotent stem cells from the HD family series and IsoHD series were cultured as described in section 2.2 prior to differentiation into MSNs using the Arber et al protocol that has been adapted as follows (section 2.3). In order to fully characterise the cells produced by this protocol, it was necessary to complete multiple optimisation steps for each experiment, which are described and presented in full in this chapter. Briefly, in order to decrease the amount of time required to fully differentiate cultures, methods of cryopreserving partially differentiated neurons were developed (section 2.3.3). To increase the material generated per differentiation and optimise the culture density and distribution for imaging experiments and electrophysiology,
additional passages were introduced at later time points of the differentiation protocol (section 2.3.4).

To ensure each experimental technique produced the most accurate and reliable data, several parameters were optimised. For example, the immunofluorescence protocol was optimised in terms of seeding density and plating method as well as the way in which cells were fixed. Each antibody used was optimised for permeabilisation protocol and antibody dilution, the results of which are described in this chapter. Once fully optimised, immunofluorescent characterisation was completed. Characterisation of the MSNs was carried out using phase contrast microscopy and confocal microscopy, detailed methods of which are in section 2.5. For image capture completed with the HCI platform, adjustments were made to the culture protocol as detailed in section 2.5.2. Analysis was carried out using Columbus cloud-based software (version 2.8.0) (section 2.5.2.3). Confirmation of dendritic spines was achieved using super resolution microscopy as described in section 2.5.3.

To assess the emergence of mHTT-associated phenotypes, a variety of methods were used. Detailed morphological analysis was completed using an IncuCyte longitudinal imaging platform as described in section 2.5.4 with in-built NeuroTrack software used for quantification. An adhesion assay previously published in the literature was used as a template for this project (Casey et al., 2015), however optimisation was carried out prior to experimentation (section 2.6.3).

The viability of MSNs at various time points in culture was assessed by running conditioned culture medium samples on a commercially available lactate dehydrogenase assay (section 2.6.1); absorbance values were normalised to internal controls for each experiment. To assess the impact of BDNF withdrawal on MSNs in both acute and chronic conditions, a protocol was developed as described in section 2.6.4. The electrophysiological properties including membrane properties and firing ability of MSNs was assessed by whole cell patch-clamp as described in 2.9. Quantification and analysis was completed by the Schorge laboratory. Statistical analysis was completed as described in section 2.11.

### 3.4 Contributions

- **Yichen Qui** performed the patch-clamp experiments and analysis presented here. Experiments and analysis were performed in blind conditions, with un-blinding completed prior to data insertion into this thesis. These experiments were performed in collaboration with Dr Stephanie Schorge and were completed under the supervision of Dr Gabriele Lignani. Experimental procedures, timelines and materials were discussed between this author and Yichen Qui for all experiments completed. The method of analysis and quantification was also discussed prior to being performed, however had to be completed by Yichen due to software licensing limitations.

- **Dr Edward Smith** performed and produced the images presented here by super resolution microscopy.
Dr Alison Wood-Kaczmar and Dr Ralph Andre first adapted the Arber et al protocol for HCl of MSN cultures and produced the original scripts for image analysis of well sets L and N, which were modified for the purpose of these experiments.

3.5 Results

3.5.1 Adaptation of PSC-derived MSN differentiation protocol and analysis methods

3.5.1.1 Cryopreservation of NSCs

The differentiation protocol to generate MSNs spans a minimum of 36 days in culture, so steps were taken to design and optimise a cryopreservation procedure that could be implemented part-way through the differentiation process. Two protocols were designed based on cell passaging techniques employed in both differentiation protocols used in this project (Arber et al., 2015a; Shi et al., 2012). Both techniques produced a heterogeneous population of viable cells, however cell viability and density was improved when the EDTA-based freezing procedure was used (Figure 3.1).

Cryopreservation of PSC-derived NSCs had not been previously described in the literature, therefore immunofluorescence experiments were designed to investigate the cell-type of thawed cells. The neuronal marker, MAP2, the MSN marker, CTIP2, the NSC marker, nestin, and the proliferating cell marker, ki-67, were used on cells that had been fixed 3-4 days post-thawing (d23-25 post-differentiation). Cells co-expressing nestin and ki-67 were considered NSCs. Cells expressing MAP2 were considered neuronal and if found to co-express CTIP2 were considered immature MSNs. Figure 3.2 clearly demonstrates that thawed cells give rise to a mixed population of both NSCs and immature MSNs, which went on to produce a heterogeneous culture of DARPP-32+/CTIP2+ neurons at d36, sample images of which can be observed in Figure 3.7. This indicated that the freeze-thaw procedure did not overtly alter the culture composition at this developmental stage and was a viable method to facilitate synchronising differentiations of multiple PSC lines.
Figure 3.1. Brightfield images of thawed MSN cultures that were cryopreserved using different methods at day 19-21 and then revived. At day 19-21 of MSN differentiation, two methods of cryopreservation were tested. Thawed cells of 22Q and 75Q HD family series successfully revived after Accutase and EDTA-mediated dissociation and cryopreservation, however the morphology and maturity of cultures appeared to differ depending on cryopreservation method. Scale bars represent 50 μm.
Figure 3.2. Confocal immunofluorescence images of revived cultures following different cryopreservation protocols. The composition of cultures post-revival was assessed by immunofluorescence to identify if the cryopreservation process would affect cell fate. Cultures of 22Q and 75Q lines were immunostained using antibodies against the proliferation marker, ki-67, the NSC marker, nestin, the MSN marker, CTIP2, and the neuronal marker, MAP2. Nuclei are shown in blue. Both methods produced a mixed culture of proliferating NSCs and maturing MSNs. Scale bars represent 50 μm.

3.5.1.2 Accutase passage of differentiating cultures
In order to generate immunofluorescent images of high quality, it was essential to produce cultures that when seeded allowed easy identification of individual cell subtypes. However it became apparent that this could not be reliably achieved with the clones tested when using the established protocol as published. The maturing MSN-containing cultures adopted a topology termed ‘ball and cabling’, whereby numerous cell bodies organised into clusters (balls) and over time formed bundles of axonal projections between clusters located nearby (cables). This cellular distribution precludes straightforward imaging and quantification, and so a third passage step was incorporated into the established protocol akin to that used in Shi et al (2015) for cortical neurons. An Accutase passage completed at day 30 was used to generate a single-cell suspension that allowed for more control over plating density and distribution.
3.5.1.3 Geltrex coating of culture plates

Following the adaptation to the established protocol described above, a second adaptation was designed and optimised by members of the Tabrizi lab. This aimed to further improve the cellular distribution of MSNs at the final passage, as well as maintain an even distribution throughout the maturation process, so that imaging experiments on aged cultures could be successfully conducted. A side-by-side comparison was conducted of cellular distribution over time after the final passage, whereby MSN-containing cultures were plated onto the advised substrate (PDL and laminin [PDL/L]), or Geltrex. Initially, it was found that the distribution of cultures immediately after passaging did not differ between substrates, however only Geltrex was able to maintain an even distribution when time in culture was extended; cultures passaged onto PDL/L displayed evidence of ‘ball and cabling’ (Wood-Kaczmar & Andre et al., under review). For this reason, all imaging experiments were conducted on MSN-containing cultures passaged onto Geltrex at d30.

3.5.1.4 d30+ passage

During aging in culture, it was observed that a population of NPCs persisted and sometimes overgrew cultures, despite including an additional passage at d30. As such, whether Accutase passages completed at a later time-point could reduce the NPC population to obtain a purer neuronal population and preserve MSNs was tested. Parallel cultures were subjected to an Accutase passage at days 35, 40, 45 and 60 post-differentiation, with observational analysis of culture viability as the primary aim and cell distribution as a secondary one. It was observed that during the d60 passage the population of NPCs could be drastically reduced, whilst seemingly preserving the neuronal population. A potential explanation for this could be that the reduced NPC population favours the differentiation of remaining NPCs, and also increases the availability of media components to post-mitotic neurons, thus generating a healthy, more mature culture. Likewise, to a lesser extent, the volume of NPCs was also reduced in cultures passaged at d35-45. Due to the timeline in which experiments were routinely completed (usually d36 ± 3 days), these additional passages were of more use when ageing was required.

3.5.1.5 Preparation of cultures for Immunofluorescent imaging

Most of the antibodies used in this study had not been previously tested in the laboratory, so each stage of the immunofluorescence protocol (section 2.5) was optimised. It was previously observed that when GFP-transfected MSNs were fixed with 4% paraformaldehyde (PFA) in PBS, the GFP signal was quenched. Therefore, subsequent fixation was completed using 10% formalin. Although this formulation also contained PFA, it was not found to quench fluorescent signal, potentially due to increased stability of the fixing solution. Furthermore, the duration of time cells spent in fixative was also found to affect the specificity of some primary antibodies binding to their epitopes. Fixing cells for a duration in excess of 15 min at RT was found to cause non-specific binding of anti-CTIP2 to a cytoplasmic epitope; CTIP2 is a nuclear protein. This phenomenon was also found to occur with other nuclear proteins such as the neuronal marker, NeuN. For this reason, the duration of subsequent fixation experiments was limited to a maximum of 15 min for standard confocal imaging, or 12 min for HCl to account for the extra
time required for formalin removal and washing. This reliably produced the correct staining pattern for all antibodies used, in accordance with the manufacturer's quality control data.

3.5.1.6 Antibody optimisation

To facilitate optimal binding of antibodies to the correct epitopes, several other parameters were optimised for confocal imaging. The first related to permeabilisation of fixed cells prior to incubation with primary antibodies. Several of the antibodies tested were targeted against transmembrane proteins with epitopes located on the extracellular surface e.g. (anti-DRD1), therefore it was hypothesised that the permeabilisation of the plasma membrane may impede optimal binding due to changes in the structure of transmembrane receptors or dispersion of epitope-binding sites. Therefore, a side-by-side comparison was conducted on cells that had either undergone a 15 min permeabilisation step, or not, prior to primary antibody addition. Whilst permeabilisation was deemed essential for some targeted proteins such as the synaptic markers PSD-95 and synaptophysin, transmembrane proteins including DRD1 were found to present with an enhanced staining pattern when the plasma membrane of cells remained intact (Figure 3. 3).
Figure 3. 3. Testing permeabilisation of cells for using antibodies for key neuronal markers. Cultures of a 22Q line were aged to d36 and fixed for immunofluorescence staining. Sister cultures were used, some of which underwent 15 min permeabilisation in 0.2% Triton-X 100 in PBS at RT, whilst the other was exposed to PBS only. Both cultures were then blocked and probed for anti-DRD1, anti-synaptophysin, anti-PSD95, and anti-MAP2 at dilutions previously described. The cells were washed and incubated with appropriate secondary antibodies prior to visualisation by confocal microscopy at 40x magnification. The staining pattern observed for DRD1 was enhanced in cells that remained un-permeabilised, whereas synaptic antibodies only displayed the expected staining pattern after permeabilisation. Scale bars represent 20 μm.

Finally, extensive optimisation was carried out for each antibody used to determine the optimal dilution that produced a high signal-to-noise ratio and minimal background (non-specific) staining. Dilution ranges were calculated as per the manufacturers’ recommendations for each primary antibody, typically three dilutions were tested on parallel cultures, accompanied by a secondary only control. An example of the dilution optimisation process can be found in Figure 3. 4. The optimised dilutions for antibodies used in subsequent experiments are listed in Table 4.
Figure 3.4. Example of antibody dilution optimisation process performed for each antibody used within the project. PSC-derived MSNs were cultured for 36 days prior to fixation. A series of antibody dilutions were carried out on sister cultures to identify the dilution that gave the best staining pattern with as high signal-to-noise ratio as possible. Secondary only controls were also completed to identify any cross-reactivity. Images were produced by confocal microscopy at 40x magnification. Scale bars represent 20 μm.

3.5.2 Confirmation of MSN identity following differentiation of PSCs

3.5.2.1 Generation of neural stem cells

The classic morphology of NSCs is a 'paving stone'-like morphology with dense cytoplasm that gives cultures a phase-dark appearance (Georgiou-Karistianis et al., 2013). Brightfield images were taken of cultures after the second passage, when a high proportion of differentiating NSCs were expected. A panel of example images can be found in Figure 3.5, showing no obvious difference between genotypes.
Figure 3.5. Brightfield images of PSC-derived cultures aged to d21 post-differentiation to an MSN fate. Using brightfield illumination at 10x magnification, PSC-derived MSN-containing cultures were observed mid-way through differentiation (d21). There was no obvious difference in morphology or maturity between genotypes; neuritic processes can be observed beginning to protrude, and somal size appears decreased, indicative of immature or transitioning neurons, indicated by white arrows. Scale bars represent 50μm.

3.5.2.2 Generation of medium spiny neurons

The addition of trophic factors BDNF and GDNF, plus maintained exposure to Activin A, aids the continued differentiation and maturation of cultures into MSNs. The brightfield images taken at ~d36 showed a reduced somal size and extensive neural processes some of which are extensively branched. Although culture density appeared higher in 22Q cultures in comparison to 75Q, because cultures were not plated at a specific density unless for specific experiments
the density differential could be due to random differences in seeding. No obvious morphological differences were observed on this scale (Figure 3.6)

![Image of clone 1, clone 2, and clone 3 of 22Q and 75Q PSCs differentiated to d36 and imaged using 10x brightfield magnification]

Figure 3. 6. Brightfield images of d36 HD family series PSC-derived MSN-containing cultures. 22Q and 75Q PSCs were differentiated to d36 and imaged using 10x brightfield magnification to capture overall culture morphology. Each clone presented with a classical neuronal morphology and extensive branching was observed. No morphological differences could be observed between genotypes, although culture density did appear to differ. Images are representative of d36 cultures and scale bars represent 50 μm.

3.5.2.3 Expression of key MSN identity markers

The classic identification of MSNs is based on morphology, as well as dual expression of DARPP-32 and CTIP2 (Arber et al., 2015a; Carri et al., 2013; Victor et al., 2014). The PSC-
derived MSNs used in this project were assessed for their expression of these markers alongside the pan-neuronal marker, βIII-tubulin as shown in Figure 3. 7. A proportion of cells in cultures of all genotypes co-expressed these markers. The exact percentages were quantified using HCI, as described below.

Figure 3. 7. Representative images of DARPP-32+ CTIP2+ MSNs. 22Q and 75Q PSC-derived MSNs were cultured to d36 prior to fixation and probing with MSN identity markers anti-DARPP-32 and anti-CTIP2 alongside a broad spectrum neuronal marker. Within each culture, a proportion of cells positively co-expressed DARPP-32 and CTIP2. Images were acquired by HCI (Opera Phenix, Perkin Elmer) at 40x magnification; scale bars represent 50 μm.

The average percentages of cells within a culture expressing key MSN identity markers was assessed by HCI and unbiased analysis. Using unique scripts written and optimised for each well set, it was possible to quantify and then compare expression levels of markers between genotypes at d36 (Figure 3. 8). To assess the neuronal purity of cultures, the percentage βIII-tubulin expressing cells (over a threshold) was assessed across cultures (Figure 3. 8A). It was observed that in a mHTT-dependent manner there was reduced neuronal purity of cultures. When compared against control (comprising the combined data sets of 22Q and 30Q lines), 45Q and 75Q cultures exhibited 34% and 30.1% reductions in neuronal purity, respectively (p = 0.0297, p = 0.0162). The identity of non-neuronal cells (~40% in control cultures) was not able to be determined.

Depending on the origin of PSC (HD family or IsoHD) there was high variability in the percentage expression of some markers. For example, the percentage of neuronal cells (as quantified by thresholded βIII-tubulin expression) that expressed DARPP-32 appeared to greatly differ between HD family and IsoHD-derived cultures: 22Q and 75Q cultures contained 13% and 14% positive expression respectively, whilst 30Q and 45Q cultures contained 2% and 1.8% respectively. This however could be due to the number of cultures tested, as a single clone was used for ESC-derived cultures, whereby the 22Q and 75Q were averages of three clones per genotype. To control for these differences, 22Q and 30Q data sets were combined and termed
'control' so that the power of comparison was increased. When assessing controls against either 45Q or 75Q cultures, no significant difference was found in the percentage of βIII-tubulin+ cells expressing DARPP-32 (p = 0.2323, p = 0.55) (Figure 3. 8B). In contrast, there was a significant difference in the proportions of CTIP2+ cells between genotypes. Percentages of CTIP2+ cells were 41.62%, 12.9% and 28.5% respectively for controls, 45Q and 75Q lines (Figure 3. 8C). These results were statistically significant (control vs 45Q, p = 0.0127; control vs 75Q, p = 0.043). As a result of the variation observed in Figure 3. 8B, the percentages of dual expression are skewed, however it should be observed that the percentages of cells co-expressing DARPP-32 and CTIP2 are nearly identical to those of DARPP-32 alone, suggesting that the majority of DARPP-32+ cells were also expressing CTIP2 (control = 7.78%, 45Q = 1.35%; p = 0.19, 75Q = 9.8%; p = 0.6) (Figure 3. 8D).

To better gauge the expression of DARPP-32, an intensity analysis was conducted (Figure 3. 8E). It was shown that there was a mHTT-dependent reduction in DARPP-32 intensity across cultures (control = 6.73 x10⁷, 45Q = 2.55 x10⁷, 75Q 2.98 x10⁷; control vs 45Q, p = 0.0056; control vs 75Q, p < 0.0005).

Finally, the proportion of cells in culture that were proliferating, or still differentiating, was determined by assessing ki-67+, or nestin+ cells (Figure 3. 8F,G,H). The levels of proliferation in culture differed depending on genotype (control = 37.31%, 45Q = 5.5%, 75Q = 22.57%; control vs 45Q, p = 0.0008; control vs 75Q, p = 0.0059). However, no significant difference was observed in the expression levels of nestin (Figure 3. 8G), although comparison of control and 45Q expression levels showed a difference that was almost statistically significant (p = 0.062). When assessing the co-expression of ki-67 and nestin, to approximate how many of the proliferating cells in culture were NSCs, a similar pattern to ki-67 staining alone was found (Figure 3. 8H), suggesting that the majority of proliferating cells in culture are indeed NSCs (control = 29.85%, 45Q = 5.1%, 75Q = 27.28%; control vs 45Q, p = 0.002; control vs 75Q, p = 0.63).
Figure 3.8. HCI analysis of key MSN identity markers assessed in PSC-derived MSN-containing cultures at d36. The images captured by HCI were subject to automated script-based analysis. Using Columbus software (2.8.0), analysis scripts were designed uniquely to each antibody pairing (well set) and once optimised, applied to all clones that had been probed with that well set. Genotypes were pooled to generate the following: the percentage neuronal purity of total viable nuclei as identified by βIII-tubulin+ cells (A); mean percentage of DARPP-32+ cells selected from βIII-tubulin+ population (B); percentage of CTIP2+ cells of total viable nuclei (C); the percentage of βIII-tubulin+ cells co-expressing DARPP-32 and CTIP2 (D); the mean intensity of DARPP-32 staining within cells (E); the percentage of proliferating cells of total viable as identified by ki-67 staining (F); the proportion of NSCs in the culture, assessed by nestin staining (G) and the percentage of ki-67+/nestin+ cells (H). 22Q and 30Q data were treated as biological replicates, and combined as control. Data presented as mean ± SEM. Means calculated from ≥ 2 clones per genotype and ≥ 3 independent experiments (≥ 120 FOV per experiment) (N = 1; n = 1-3; n ≥ 12). Significance is shown as * p < 0.05, ** p < 0.01, *** p < 0.001, p < 0.0001.
3.5.2.4 Additional MSN identity markers

Although co-expression of DARPP-32 and CTIP2 is the ‘gold standard’ for MSN identification (Arber et al., 2015b; Delli Carri et al., 2013), there are several other key markers that are required for MSN verification. Inhibitory neurons in the CNS including MSNs should express γ-aminobutyric acid (GABA) and its receptors (e.g. GABA A receptor [GABAa]). The calcium binding protein calbindin, is highly expressed in the striatum. Furthermore, as aforementioned, MSNs comprising the direct and indirect pathway can be distinguished by their unique expression of dopamine receptor subtypes (DRD1 = direct, DRD2 = indirect); neurotransmitters (substance P = direct), endogenous ligands (enkephalin = indirect) and adenosine receptors (A1Ar = direct, A2Ar = indirect). To further confirm MSN identity and verify the expression of these alternative markers, PSC-derived MSN-containing cultures from the HD family series were aged to d60 in culture and subject to HCI and automated analysis. The choice to use the HD family series was based simply on material availability at the time of experimentation (Figure 3.9). Aged cultures were used to ensure that the cells would be expressing the proteins and receptors to be assessed; it is known that in the human brain, expression of GABA receptors is low, and peaks at 1 year of age (Webb et al., 2001).

To first identify whether viability was compromised by genotype in aged conditions, a ‘snapshot’ of culture viability of cells was assessed by counting pyknotic nuclei (Figure 3.9A), but no significant difference was observed (p = 0.155). To assess whether the total cell number was different between genotypes, and also assess changes to cell number over time, the total number of nuclei was quantified as shown in Figure 3.9B. Although no significant difference was observed between 22Q and 75Q total cell counts at d36 (p = 0.25), a significant reduction in total cells was found at d60 (5191 vs 3496, p = 0.0007). Furthermore, when assessed by two-way ANOVA, a trend to significance was found between cell counts of 75Q MSNs between days 36 and 60 (p = 0.062), whereas no difference was observed in 22Q lines (p > 0.99), suggesting 75Q MSNs have increased cell loss over time. As a measure of MSN proportion, the number of CTIP2+ neurons was assessed (Figure 3.9C) and interestingly, 75Q cultures had a significantly increased percentage of CTIP2+ cells (20Q = 13.18% vs 75Q = 38.25%; p < 0.0001). In contrast there was a significantly reduced number of proliferating cells in 75Q cultures compared to 22Q (22Q = 45.92 vs 75Q = 35.14%; p < 0.0001) (Figure 3.9D), as well as a significantly reduced population of nestin+ cells (22Q = 86.91% vs 75Q = 76.78%; p = 0.0196) (Figure 3.9E) suggesting an increased maturity within these cultures. Finally, the number of proliferating cells co-expressing nestin was also significantly reduced in 75Q cultures (22Q = 52.81% vs 75Q = 46.08%; p = 0.0258) (Figure 3.9F). As neuronal purity was not assessed at this time, it was not possible to discern whether the differences in populations are caused by a reduced neuronal purity of 22Q cultures, or perhaps these data are a result of the persistent proliferation that has been previously described. It was notable that this occurred more frequently in control cultures than those expressing mHTT, which could be a HD-associated phenotype in itself.

Assessment of the alternative MSN identity markers also showed differences. Although there appeared to be a reduction in the percentage of GABAa in 75Q cultures, this difference was not
significant (p = 0.33) (Figure 3. 9G), but assessing the mean area of GABAa+ cells showed a significant reduction in area in 75Q cultures (22Q = 51.26μm² vs 75Q = 33.6 μm², p = 0.0251) (Figure 3. 9K). Cultures expressing mHTT did have increased percentages of DRD1-expressing cells, as well as calbindin+ cells (22Q = 48.82% vs 75Q 62.10%; p = 0.0082 [Figure 3. 9H]; 22Q = 71.51% vs 75Q = 83.08%, p = 0.206 [Figure 3. 9I]), however had a slightly reduced percentage of cells expressing indirect pathway marker A2Ar (22Q = 21.58% vs 75Q = 20.64%) (Figure 3. 9J), although this result was not significant (p = 0.755), and the intensity of A2Ar staining also only trended toward significance (22Q = 487.2 vs 75Q = 538.1. p = 0.0855) (Figure 3. 9M). Finally, the proportion of calbindin+ cells that co-expressed either DRD1 (Figure 3. 9L) or CTIP2 (Figure 3. 9N) was assessed however no significant difference was found in expression levels between the two genotypes (DRD1: 22Q = 67.09% vs 75Q = 75.25%, p = 0.1; CTIP2: 22Q = 18.54% vs 75Q = 16.28%, p = 0.644).
Figure 3. 9. HCI analysis of additional MSN identity markers in PSC-derived HD family line MSNs at d60. Bar charts show the mean A) viability of cultures; B) total nuclear cell count at d36 and d60 C) percentage of CTIP2+ cells; D) ki-67+ cells; E) percentage of nestin+ cells; F) percentage of ki-67+/nestin+ G) percentage cells expressing GABAa; H) percentage of direct pathway MSNs as identified by DRD1 expression; I) percentage of calbindin+ cells; J) percentage of indirect pathway cells, as identified by A2Ar expression; K) the average area of GABAa expression (μm²) per well; L) the percentage of calbindin+ cells co-expressing DRD1; M) the average A2Ar pixel intensity per well; N) the percentage of calbindin+ cells co-expressing CTIP2. Data are presented as mean ± SEM. Data represent a minimum of 2 clones per genotype (≥80 FOV per clone) (N = 1; n = 3; n ≥ 12). * p < 0.05, ** p < 0.01, *** p < 0.001 p < 0.0001.
3.5.2.5 Dendritic spine formation in matured PSC-derived MSN-containing cultures

MSNs have a distinct morphology, giving rise to their name, that is a high density of dendritic spines populate their neuronal processes which, when observed under high magnification, give the neurons a 'spiny' appearance. Dendritic spines have a well-documented morphology dependent on their maturation stage (Korobova and Svitkina, 2010). This project attempted to confirm the presence of dendritic spines using an antibody targeted against Spinophilin, a protein enriched in dendritic spines. Although dendritic puncta were identifiable with this antibody, the reliability of staining was such that quantification was not possible. Thus, a transient transfection of GFP into maturing cultures was performed so that the entirety of the cell - including dendritic spines - was fluorescently labelled. At this point, super resolution microscopy was performed and clearly demonstrated dendritic spines of varying maturity (Figure 3.10); in some images, elongated tendrils were present which indicate an immature spine. As can be seen in the bottom panel, the majority of spines observed presented the classic 'mushroom' shaped puncta that are typically associated with mature cultures (Korobova and Svitkina, 2010). These experiments were conducted in mHTT-expressing lines, therefore it would be of value to compare dendritic spine morphology against that of control lines also.
Figure 3. 10. Dendritic spine imaging of 22Q PSC-derived MSN-containing cultures aged to 100 days. Using super resolution microscopy, images of dendritic spines and their morphology was assessed under 100x magnification. Cultures (here 22Q clone 2) were transiently transfected with GFP using lipofectamine transfection at day 70 and aged in culture to 100 days. Sample images show multiple neural processes populated with dendritic spines of varying morphology and density. A zoomed image is shown in the lower panel depicting 'mushroom' boutons, classically defined as mature dendritic spines. Scale bars represent 10 μm.
3.5.3 Mutant HTT-associated phenotypes in PSC-derived MSN-containing cultures

3.5.3.1 Cellular adhesion

During routine culture of both PSCs in their native state and those undergoing MSN differentiation, it was notable that the levels of adhesion to coating substrate appeared to differ between genotypes; mHTT expressing lines detached more easily during passaging and were also more challenging to maintain whilst ageing in culture, due to cell detachment. Furthermore, it was identified by HCl that the total cell number at both d36 and d60 were decreased, the latter significantly so, in 75Q MSNs in comparison to 22Q (Figure 3. 11B). Whilst this could be attributable to cell viability, it was hypothesised that cellular adhesion may also be compromised in mHTT expressing lines. To quantitatively assess adhesion in these cultures, an in-house adhesion assay was designed (section 2.6.3). There was a mHTT-dependent reduction in the ability of PSCs to adhere to a Geltrex substrate when assessing the lines dependent on PSC origin (Figure 3. 11A & G) i.e. HD family series or IsoHD series. Significant reductions in adhesion were found: 22Q vs 75Q p < 0.0001; 30Q vs 45Q p < 0.0001; 30Q vs 81Q p < 0.0001. When control lines of 22Q and 30Q were combined and termed 'control' (Figure 3. 11B), they exhibited significantly increased adhesion above all lines expressing mHTT [control vs 45Q, p < 0.0001; vs 75Q, p < 0.0001; vs 81Q, p < 0.0001]. A significant increase in adhesion was also found between 22Q and 30Q lines however (22Q = 89.65 vs 30Q = 144.3 mean adhered cells, p < 0.0001), suggesting potential inconsistency between lines.

To identify whether differences in adhesive potential persisted through the MSN differentiation process, the adhesion ability of NPCs to laminin substrate was assessed at d22 differentiation (Figure 3. 11C & F). When 22Q and 30Q genotypes were combined as 'control' and when compared to 75Q levels of adhesion, a significant reduction was found (p < 0.0001). A significant increase in adhesion was also found between 22Q and 30Q NPCs, interestingly in the opposite direction to PSC adhesion, with a mean difference of 50.67 cells (p = 0.006).

Finally, adhesion of d36 MSN-containing cultures to laminin substrate was assessed. When 22Q and 30Q genotypes were combined as 'control', a Student's t-test returned a significant reduction in 75Q MSNs (p < 0.0001) Figure 3. 11D & G). The increased adhesion observed in 22Q lines to 30Q was maintained in MSNs, with a mean difference in adhesion of 17.72 cells (p = 0.0059).
Figure 3. Adhesion assay quantification and representative images of PSCs, NPCs and mature MSNs. The adhesion ability of PSCs and PSC-derived MSN-containing cultures at different stages of differentiation was assessed using an in-house adhesion assay. Adhesion was assessed at A) PSCs stage, B) PSCs with control lines combined, C) at d22, equating to NPC predominant cultures, and D) at d36 when cultures comprised a population of MSNs. Representative bright field images of PSC cultures (E), NPCs (F) and MSNs (G) post-adhesion assay. Three FOV were manually quantified for each clone which was run in duplicate. Data are presented as mean ± SEM; control = 22Q and 30Q data combined as biological replicates (N =2; n=3; n ≥ 8) ** p < 0.01; *** p < 0.001; **** p < 0.0001.
### 3.5.3.2 Longitudinal assessment of cell viability.

To address whether the expression of mHTT affected MSN viability, and also to test whether this was dependent on developmental stage, a longitudinal assessment of cell viability was conducted. PSC-derived MSN-containing cultures aged to 25 days (Figure 3. 12A), 36 days (Figure 3. 12B) and 50 days (Figure 3. 12C) were subject to LDH assay under basal conditions. The results are challenging to interpret, as no consistent effects were found. To increase the power of the statistical tests, data obtained from 22Q and 30Q lines were treated as biological replicates and termed control. At NPC stage (Figure 3. 12A), 75Q cells had significantly reduced viability when compared to control (-12.04% viable, p = 0.0001), but no difference was observed in 45Q NPCs (-0.83% viable, p = 0.79). At d36, the viability of cultures also differed between genotypes (Figure 3. 12B): control cultures were on average 5.4% less viable than 45Q cultures (p = 0.0011), but 7.31% more viable that 75Q cultures, (p < 0.0001). An interesting result was found in MSN-containing cultures aged to d50, whereby 75Q cultures showed a significantly increased percentage of viable cells compared to control (Figure 3. 12C). On average, controls showed -5.73% viability compared to 75Q (p = 0.0024, p < 0.0001), whereas no difference was observed between controls and 45Qs (0.03% difference, p = 0.99).

![Figure 3. 12. Cell viability under baseline conditions across differentiation of PSCs to an MSN fate.](image)
PSC-derived MSN-containing cultures were subject to LDH assay assessment at different stages of differentiation and maturity. A) NPC cultures assessed at ~25; B) MSN-containing cultures assessed at the end of the differentiation protocol, at d36; C) matured MSN-containing cultures which had been aged to d50. Data from 22Q and 30Q lines were combined as 'control'. Data are presented as mean ± SEM. (N =2; n= 1-3; n ≥ 12). ** p < 0.01, **** p < 0.0001.

### 3.5.3.3 MSN viability under stress

As differences in viability had been found in differentiating and maturing MSN cultures in baseline conditions, it was hypothesised that the application of cellular stress may amplify these differences, as has been found in the literature previously (reviewed in Wiatr et al., 2018). As such, the impact of acute BDNF withdrawal, as well as chronic BDNF deficiency on culture viability was assessed. As aforementioned, BDNF is a key neurotrophic factor responsible for MSN survival and maturation in the brain, therefore, by withdrawing this neurotrophic factor, a
stress response should be activated. Chronic BDNF deficiency has not yet been studied in the literature therefore these were novel investigations into the cellular response elicited as a result.

To assess the impact of acute BDNF withdrawal on viability, PSC-derived MSN-containing cultures aged to d36 were maintained in culture media that was not supplemented with BDNF for either 24 or 48 h. After the designated withdrawal period, LDH assays were carried out on the conditioned media removed from cells (Figure 3. 13). An acute measurement was also taken at 0 h withdrawal to allow for comparisons. In the HD family series, the 0 h time-point returned no significant difference between genotypes (p = 0.4531) (Figure 3. 13A), however a significant increase in viability (mean = 3.9%) was found between 45Q cultures when compared to the 30Q line of the IsoHD series (p = 0.0019). In contrast no change was found between 30Q vs 81Q cultures (p = 0.237) or 45Q vs 81Q cultures (p = 0.177) (Figure 3. 13B).

After 24 h BDNF withdrawal, a small but significant mHTT-dependent effect on viability was found, with the HD family series exhibiting a 2.2% difference in viability between 22Q and 75Q cultures (p = 0.093) (Figure 3. 13C), which was complimented by a similar result in the IsoHD series (Figure 3. 13D): overall significance p < 0.0001; 30Q vs 45Q, p = 0.0023; 30Q vs 81Q, p < 0.0001. When assessed after 48 h, although no significant difference was seen in the HD family series (p = 0.18) (Figure 3. 13E), a significant reduction in viability was maintained (overall p = 0.0002) in the 81Q IsoHD series, when compared to both 30Q (p = 0.0003) and 45Q cultures (p = 0.001) (Figure 3. 13F).
Following on from the mHTT-dependent phenotypes described above, a series of experiments were designed that would assess the impact of chronic BDNF deficiency on culture viability. Indeed, one aim here was to identify whether cultures that had never been exposed to BDNF during differentiation and maturation would have compromised viability compared to cultures that had had the normal exposure. As such, at d26 of the differentiation protocol - the time-point at which BDNF is normally introduced - parallel cultures were established in which half received...
fully supplemented culture media (MSN post-26 + Activin A, GDNF and BDNF) in which they were maintained throughout the experimental timeline, and half received culture media supplemented only with Activin A and GDNF, thus these differentiating MSNs were never exposed to BDNF. Conditioned media samples were then taken to be assessed after 4, 14 and 24 days by LDH assay, so that a longitudinal assessment of viability was possible in +/- BDNF cultures (Figure 3. 14).

At d30 of differentiation and four days of +/- BDNF, a heterogeneous culture of NPCs and MSNs was generated and HTT polyQ-length produced 13.87% of variation overall (p < 0.0001). However, when corrected for multiple comparisons, there was no significant effect on cell viability after four days BDNF deficiency (Figure 3. 14A). After 14 days of +/- BDNF, at d40 differentiation, cultures were still heterogeneous, but had a higher proportion of MSNs, which have previously shown vulnerability upon BDNF withdrawal both in this project, and the literature (Mattis et al., 2015). However, the data shown in Figure 3. 14B suggests this may not be the case. The HTT polyQ-length accounted for 24.45% total variation in viability (p < 0.0001); in 45Q cultures treated with BDNF, there was a trend toward increased viability when compared to control (p = 0.09), but no difference was found in BDNF deficient comparisons (p = 0.126). A significant reduction in viability was observed between 75Qs and control in both + BDNF (p < 0.0001) and - BDNF (p = 0.0004) conditions. No significant differences were observed between control and 81Qs in either + BDNF or - BDNF conditions. Overall treatment as a variable did not have a significant directional effect on outcome (p = 0.7276).

At d50 assessment, and after 24 days +/- BDNF, the results show that cultures that had never been exposed to BDNF had enhanced viability, independent of genotype or mHTT status. Remarkably, 75Q cultures had enhanced viability above control (Figure 3. 14C). At this time point, there was a significant interaction found between treatment and HTT polyQ-length (p = 0.0148), as well as polyQ-length and treatment contributing 20.02% and 7.265% variation respectively (p < 0.0001, p = 0.0006). In cultures exposed to BDNF, no significant difference was found between controls and 45Qs (p > 0.999), however a significant increase in viability was observed in 75Q cultures with BDNF treatment (mean survival 84.3% vs 90%, p < 0.0001). In BDNF deficient conditions, no significant differences were found between controls and HD genotypes (45Q p > 0.99, 75Q p = 0.16).
Figure 3. Chronic BDNF deficiency in PSC-derived MSN-containing cultures spanning d26 - d50 in culture. Parallel MSN cultures were either exposed to BDNF (10 ng/ml) from d26 onward, or not. Cell viability was assessed at A) d30 (BDNF deficient = 4 days), B) d40 (BDNF deficient = 14 days), or C) d50 (BDNF deficient= 24 days). Data are presented as mean ± SEM. Data obtained from 22Q and 30Q genotypes were treated as biological replicates and termed control. (N =2; n=1-3; n ≥ 12). ***, p < 0.001; **** p < 0.0001.

3.5.3.4 Membrane properties
The electrophysiology of PSC-derived MSNs has been investigated before and can give an indication into the maturity of cultures (The HD iPSC Consortium, 2012). Previous reports have
suggested that MSNs expressing mHTT have a delayed maturation rate, however other groups have found hyper-excitability, or an inability to fire action potentials. Here, using a whole-cell patch clamp technique, this project investigated various aspects of MSN electrophysiology and membrane properties, including: cell capacitance, input resistance, the threshold current or input current and maximum number of action potentials fired by the same cell per recording session (Figure 3. 15). Whilst the data were variable, potentially due to the maturity or identity of the patched cell, some trends were observed between genotypes. The cell capacitance, which gives an indication of membrane size and therefore cell size, was slightly reduced in mHTT expressing lines, but was not significant (p = 0.073). When Bonferroni corrected, the most significant result was 22Q vs 81Q (mean difference 89.4pF, p = 0.091) (Figure 3. 15A). Conversely, input resistance which indicates how many channels are open on the membrane (the higher the resistance, the less current is conducted across the membrane) increased with increasing HTT poly-Q-repeat length, suggesting a lack of open channels on mHTT-expressing cell membranes, this result however was not considered significant (p = 0.55) (Figure 3. 15B). The latency to fire - MSNs exhibit a delayed latency to fire the initial action potential - was equivalent across groups (Figure 3. 15C). The required input current to generate action potentials from cells was also variable across genotypes. Although the current required to elicit an action potential in 75Q and 81Q cultures, was much less than 22Qs (60pA and 42pA respectively, compared to 125pA), 30Q cultures required on average 25pA, and 45Qs were more comparable to 22Q cultures, requiring 116 pA. Indeed, the stark contrast between control lines trended toward significance (p = 0.08) (Figure 3. 15D). Finally, the mean number of action potentials generated from single cells was calculated (Figure 3. 15E), however no overt differences were found (p = 0.187). In an effort to distinguish if cell origin (HD family or IsoHD series) had an effect on the results, preliminary statistical analysis was completed comparing lines from the same origin i.e. no cross-comparison between HD family series and IsoHD series. The only difference to the results described previously, was a significantly increased input current in 45Q cultures compared to 30Q (p = 0.0286); there was no significance found in any other comparison.
Figure 3. 15. Membrane properties and action potential firing in PSC-derived MSN-containing cultures. A variety of membrane properties were assessed in PSC-derived MSNs aged to d65 in culture, using whole-cell patch clamp. The cell capacitance (pF) (A), input resistance (MΩ) (B), delayed latency to fire (s) (C), threshold current (pA) (D) and mean number of action potentials elicited per cell per recording (E) were assessed. Data are presented as mean ± SEM (N = 1, n = 1-2; n = 4-10).

3.6 Discussion

Neurons of the striatum, in particular the MSNs that comprise 95% of the brain region, are known to be particularly vulnerable in HD, and exhibit pathology throughout the progression of the disease before they ultimately degenerate and die. The mechanism behind this selective vulnerability to mHTT expression is still yet to be fully elucidated, despite extensive investigation in multiple species (Huang et al., 2016; Vonsattel et al., 2011). As HD is a human disease, it is plausible that the answer to this phenomenon may only be revealed in human studies. With the advent of PSC-derived neuronal studies, we can now investigate previously uncharacterised aspects of human neuronal biology in HD.

In order to begin investigating HD-associated phenotypes within the PSC-derived MSN-containing cultures used here, it was necessary to complete a characterisation of the cellular cohort to ensure the MSNs generated by the modified Arber et al protocol were typical of those found in vivo. Immunofluorescence was used to assess culture composition prior to an investigation into the functionality and phenotypes of PSC-derived MSN-containing cultures expressing mHTT. Investigations focused on differentiation ability and maturation, as well as cellular adhesion, viability with or without external stressors, and the electrophysiological properties of cultures. The data produced includes novel findings as well as those that support or contradict previous studies.
3.6.1 PSC-derived MSN characterisation and maturity

There are several protocols than can be employed to generate MSNs from PSCs, however this project utilised the Arber et al protocol, previously tested in our laboratory and found to reliably generate cultures containing MSNs within 36 days (Arber et al., 2015b). To further adapt the protocol for the experiments required for this project, modifications were introduced and optimised which enabled more control over plating density and distribution at experimental endpoints.

Initial characterisation was focused on identifying whether both the HD family series and IsoHD series were able to reliably differentiate into DARPP-32+/CTIP2+ neurons, which would indicate MSN identity. Although the percentages of cells varied between genotypes and lines, all PSC lines were able to successfully produce a proportion of MSNs within 36 days. This is somewhat controversial in the literature, as although there are several publications showing that differentiation potential and efficiency is not compromised by mHTT (Nekrasov et al., 2016; Xu et al., 2017b), others report a delayed maturation rate resulting in a reduced differentiation efficiency in mHTT-expressing lines compared to controls (Conforti et al., 2018). It should be noted however that the methods of assessment differed between studies. Conforti et al. completed a step-wise study in which culture composition was assessed at multiple time-points. The authors found that mHTT-expressing PSCs undergoing striatal differentiation retained the pluripotency marker OCT4, and cells struggled to acquire neuroectodermal fate. Also, at later time points (d30 differentiation) a reduced percentage of CTIP2+ cells were observed, which the authors attribute to a slower migration into the marginal zone and thus delayed maturation (Conforti et al., 2018). These experiments were completed however, with cell lines expressing very high HTT polyQ-lengths therefore may not be as applicable to more physiological repeat sizes typically found in the HD population.

Whilst no significant difference was observed in the percentage of DARPP-32+ cells in this cohort, there was a significant reduction in CTIP2+ mHTT-expressing lines at day 36, suggesting this may be a consistent phenomenon. However, this project did not complete a longitudinal analysis of culture composition, so from these results at least, it cannot comment on whether the reduced CTIP2+ percentage is due to altered patterning, or delayed maturity; this would be an interesting aspect to examine further. The identity of these CTIP2+ / DARPP-32-cells is also call into question, as one suggest they are MSN-precursors however this was not confirmed.

The proportion of neurons within cultures also differed between genotypes in a mHTT-dependent manner. This is also controversial in the field of PSC-derived neuronal studies, as there is evidence for both up-regulated (Guo et al., 2013; Lorincz and Zawistowski, 2009; McQuade et al., 2014) and down-regulated neuronal purity upon differentiation completion, in terms of the percentage of cells expressing neuronal markers such as βIII-tubulin or MAP2 (Conforti et al., 2018). A theory described in Wiatr et al., (2018) suggests that the HTT poly-Q-repeat length may influence this, with juvenile HD repeat lengths documented as having increased neuronal purity, compared to adult-onset lengths, which exhibit reduced neuronal
purity. A potential explanation for this may arise from the evidence that NPCs are vulnerable to mHTT toxicity. It is plausible that with increasing HTT polyQ-length (as seen in juvenile cases) the population of NPCs within cultures decreases at an earlier time point, thus resulting in a higher neuronal purity (Wiatr et al., 2018). The data presented here showing a polyQ-length decrease in viability at d30 supports this.

Anecdotally, during the course of routine culture, it was observed that proliferation of precursors was greater in control cultures than HD. Indeed, when assessing proliferation rates as well as the proportion of NSCs within cultures, the data clearly showed that MSN cultures expressing mHTT had reduced proliferation and NSC populations compared to controls, especially when assessed in more mature cultures. This result corroborates that found in Conforti et al. and Mattis et al., as well as several studies completed in murine cellular studies (Wiatr et al., 2018). However, no difference was observed in proliferation rates of NSCs or NPCs in multiple other studies (Camnasio et al., 2012; Castiglioni et al., 2012). A possible reason for this could be due to the experimental technique used to quantify proliferation. One must also not overlook the fact that small changes in culture environment could also influence the culture composition and therefore phenotype.

The finding that CTIP2+ neuronal populations increase simultaneously with a reduction in proliferating cells in aged mHTT-expressing cells, suggesting HD lines are somehow ‘more mature’ poses an interesting question. In the human brain, the toxic effect of mHTT is cumulative, and one would assume this could also be the case in culture; although mHTT positive aggregates are not regularly found in PSC-derived cultures, this does not rule out the possibility of free-floating monomeric mHTT within cells that can still exert toxic effects. These data could be suggesting that it is in fact the immature cell types, such as NSCs and NPCs which are more susceptible to mHTT-induced cell death, whilst more mature neuronal cell types are resistant - a hypothesis also drawn from previous publications (Jeon et al., 2012; Mattis et al., 2015).

The question arises therefore, as to why there are such diverse and often contradictory findings as to the effect of mHTT on PSC-derived MSN cultures. With each cellular composition marker discussed above, there are conflicting results in the literature, and there are several possible explanations for this. A large proportion of published studies were completed with commercially available clones of 60, 109, and 180 HTT CAG-repeats, the latter being lines with extremely long repeat lengths that are most likely to give the strongest phenotypes. Comparing alongside more common allelic lengths is likely to give variable data as phenotypes from shorter repeat lengths may tend not to emerge as early or be as severe. In addition, there is an emergent theory that mid-length alleles, such as around 50 CAGs, in mice at least, confer beneficial effects such as increased cell division, a concept known as antagonistic pleiotropy (Morton et al., 2019). This could in turn produce inconclusive results in terms of the early effects of mHTT on differentiating neuronal cultures. In addition, in many cases detailed above, data has been obtained from a single clone per genotype, which does not allow the authors to control for potential off-target effects caused by somatic cell reprogramming itself (Conforti et al., 2018; HD
None of these studies included isogenic controls, and as the PSC lines were derived from non-related individuals, it is plausible that differences in genetic background between controls and HD lines could be confounding the emergence of clear-cut, robust phenotypes.

Secondly, the differentiation protocols used to generate MSNs differ in their timescales, physical interventions (passaging), and chemical exposure or trophic support. It is therefore challenging to make comparisons between studies as these factors are likely to have important biological effects on differentiation and cell viability – as already reported from optimisation experiments within this project. It is for these reasons that this project was completed with both iPSC- and ESC-derived MSNs, subject to the exact same differentiation protocol. Furthermore, the use of PSC-lines derived from related individuals (HD family series) acts to minimise genetic background variability to an extent (50%), which is of course not a contributing factor in the IsoHD series. Finally, as all experiments were conducted (where possible) with multiple clones of the same genotype, this controls for the effect of reprogramming on the PSC clones, adding an additional level of experimental control, these measures taken together will have increased the robustness of the assays and simplified interpretation of the data.

Basal ganglia circuitry is comprised of two pathways with regards to MSNs, termed the direct and indirect pathway. MSN populations are categorised according to their innervation pathway, positive or negative regulation of the substantia nigra and thalamus, as well by the kinds of neurotransmitters, proteins and receptors they express (Matamales et al., 2009). The Arber et al protocol provided evidence that MSNs of both the direct and indirect pathway are generated however the proportions of each in culture were not quantified. Using antibodies targeted against DRD1, and A2Ar, it was possible to estimate the percentage of MSNs that were categorised into the direct and indirect pathway, respectively. Due to limitations of host antibody species, unfortunately these populations could not be assessed within the same experiment, and thus were limited as quantification had to be carried out on parallel cultures. This could also explain how the combined percentages of DRD1+ and A2Ar+ MSNs exceeds 100%. There is also a subpopulation of MSNs within the human brain that co-express direct, and indirect markers, therefore this could also be a contributing factor. Irrespective of these caveats, an increased proportion of DRD1+ neurons was found in mHTT-expressing cultures, which has important implications for HD-associated phenotypes discussed at a later point. It is well known than the MSNs of the indirect pathway degenerate prior to direct pathway MSNs in HD, therefore it is also of interest that the intensity of A2Ar+ cells was reduced in 75Q MSNs whilst percentage of DRD1+ MSNs was elevated. This provides tentative evidence that the degeneration observed in the human brain i.e. indirect pathway > direct pathway degeneration, may also be occurring in in vitro conditions.

The development of complex dendritic arborisations and spines is required by most neurons in order to receive and process information within the brain and is thought to be how plasticity in the brain manifests (Murphy et al., 2000). This process is particularly pertinent in MSNs, therefore the data presented here that provides evidence of abundant dendritic spine formation
albeit in various states of maturity, is positive. Dendritic spines are extremely dynamic structures that change their morphology in line with synaptic activity, hence conferring the property of plasticity (Risher et al., 2014). High levels of synaptic activity result in a remodelling of the actin cytoskeleton which causes dendritic spines to exhibit the classic 'mushroom' shape, accounting for the majority of spines observed in these cultures. However, when synaptogenesis is occurring, or when synaptic activity drops, the actin cytoskeleton is remodelled to form lamellipodia-like structures, therefore the presence of these 'immature' spines in cultures suggests they are dynamic in their synaptic activity (Korobova and Svitkina, 2010). In the Arber et al protocol, spine morphology was identified by DARPP-32 immunofluorescence, as well as Alexa-Fluo 488 whole cell fill. Although these methods were trialled by this project, as well as co-labelling with an antibody targeted against dendritic spines (Spinophilin), the resolution required to clearly observe spine morphology was not sufficient, hence super resolution microscopy was utilised. However, this is a very time consuming and low-throughput technique and here only a small sample of control MSNs were assessed. As such, it is unknown if the frequency or morphology of dendritic spines were altered in the presence of mHTT; it would be of interest to complete a direct comparison as this could also indicate the level of maturity in cultures.

3.6.2 Functional HD-associated phenotypes in mature MSN-containing cultures

3.6.2.1 Cell viability is affected by culture composition and age

Once full characterisation was complete, investigations could begin into the emergence of HD-associated phenotypes in culture. As MSNs are the most vulnerable cell type in the disease, initial studies were first conducted into cell viability in culture.

There are conflicting reports regarding the viability of HD MSNs under baseline conditions, without stress or stimulation. This project identified that viability was affected by numerous factors including primary composition of the culture and its maturity. A mHTT-dependent reduced viability phenotype was observed in NPC cultures at day 30 which persisted in d36 cultures comprising predominantly neurons (a 3:2 ratio of neurons: NPCs). However, in more mature cultures, whilst an HD-associated viability phenotype was also observed, the opposite effect was found, with mHTT-expressing MSNs showing enhanced viability compared with non-HD lines. Interestingly, the viability of the HD family series increased with age, this would suggest that it is the more immature cell types that are more sensitive to mHTT induced toxicity, such as NSCs and NPCs.

The viability of MSNs in HD models is somewhat controversial, as in knock-in HD animal models for example, neuronal loss is not seen in spite of symptom manifestation (Menalled, 2005). Similarly, previously published studies using PSC-derived MSNs have failed to find a cell death phenotype in baseline conditions, with compromised viability only observed after the application of external stressors (reviewed in Wiatr et al., 2018). A possible explanation for the discrepancy in phenotype observed in this study, could be related to the genetic background of the cell lines used. As aforementioned, the cell lines used in published studies largely employ
commercially available lines with different genetic backgrounds. As HD-associated phenotypes have not yet been studied in PSCs derived from related individuals, these data could suggest that genetic variation confers a bigger impact on phenotype than previously thought. Furthermore, when genetic background is identical (as in isogenic lines), an increase in viability was observed in acute conditions in adult onset HTT CAG-repeat lengths, but not juvenile onset. Combined, these data suggest that there may be a compensation mechanism in mHTT-expressing cells which can accommodate the toxicity of mHTT to an extent. Indeed there has been evidence presented both from murine models of HD as well as non-neuronal patient cells that autophagy mediated degradation is increased in early-HD, however declines with age and disease progression (reviewed in (Cortes and La Spada, 2014). Taking this further, it is plausible that if occurring, the compensating mechanism could reach a threshold at which point it is overcome, and logically that would occur more quickly in more severe disease cases such as juvenile HD. This however is just one hypothesis, and other explanations could also be plausible.

One method to test this hypothesis, was to expose cultures to exogenous stress, which in theory should reduce the compensation threshold and result in an HD-associated phenotype. A common method of inducing cellular stress is to withdraw neurotrophic factors important for MSN functionality, such as BDNF. In normal physiology, BDNF binding and activation of the TrkB receptors present on MSN dendrites results in initiation of three intracellular signalling cascades that mediate anti-apoptotic events, protein translation and synaptic plasticity (Baydyuk and Xu, 2014). Therefore, if BDNF is withdrawn, theoretically these cascades would not be activated and the opposite results, namely cell death would be more likely to occur. Multiple studies have observed a reduction in viability as a result of BDNF withdrawal in acute conditions over and above that seen in non-HD cells (Mattis et al., 2015; The HD iPSC Consortium, 2012; Xu et al., 2017b), therefore the data presented here, that MSN-containing cultures have increased susceptibility to stress-induced cell death, is in line with those studies.

Previous studies have focused on viability after acute BDNF withdrawal commonly lasting 48 h only, which encouraged a novel series of experiments in this project, as the impact of chronic BDNF withdrawal, or in effect BDNF deficiency, was yet to be investigated. An experiment was designed such that the viability of cultures that had never been exposed to BDNF, and thus were chronically deficient, was compared against cultures maintained in normal culture conditions. The results of this experiment were surprising, as it was observed that especially in later time points (24 days +/- BDNF), chronically ‘stressed’ cultures actually showed consistently higher viability across genotypes. One theory as to why this occurs relates back to culture composition, not in terms of the immature vs mature neuronal cell types, but in terms of direct vs indirect pathway MSNs. It was identified by Baydyuk et al that whilst 98% of indirect pathway MSNs (A2Ar+) expressed the BDNF receptor TrkB on their membranes, only 18% of direct pathway MSNs (DRD1+) showed expression (Baydyuk et al., 2013, 2011; Baydyuk and Xu, 2014). This suggests that MSNs of the indirect pathway would be affected more by BDNF withdrawal than direct pathway MSNs. As this project identified a higher proportion of cells expressing DRD1 than A2Ar (40-60% vs 20%), and DRD1+ cells were more numerous in 75Q
cultures, it could be argued that this may be the reason behind the maintained viability observed. Unfortunately, the expression levels of TrkB could not be easily quantified in this project; the selected antibody exhibited a high level of erroneous binding when assessed using HCl, therefore signal quality and specificity was compromised, as such this hypothesis could not be tested further.

3.6.2.2 Cellular adhesion is compromised in PSC-derived MSN-containing cultures

Any differences observed between genotypes in the routine culture of PSCs and differentiating MSNs were noted throughout the project and later investigated to identify the presence of an HD-associated phenotype. It became apparent that the adhesion of cultures differed depending on mHTT expression, thus adhesion assays were employed to quantify these differences. PSCs expressing mHTT had significantly reduced adhesion compared to control counterparts. Progressing through differentiation and maturity, the same phenotype was observed at days 25 and 36, suggesting that mHTT-expression confers a reduction in adherence potential in NPC- and MSN-containing cultures also.

It has been reported previously that multiple cell types expressing mHTT have altered adhesion in culture (Reis et al., 2011). The authors identified that striatal neurons derived from HdhQ111 mice had altered levels of both cell-cell adhesion as well as cell-substratum adhesion when plated on laminin substrate. The mechanism behind this was found to be reduced N-cadherin levels in HD samples, both in baseline conditions and after experimental energy depletion. N-cadherin is required for cell-cell and cell-substratum adhesion as well as neurite outgrowth and other dynamic cellular process, therefore when depleted these processes are compromised. Similarly, when replicating the experimental set-up from the previous publication, the HD iPSC Consortium identified that human PSC-derived NPCs expressing either 60 or 180 HTT CAG repeats demonstrated altered adhesion via actin-binding and reduced cell-cell adhesion resulting in reduced cell clustering (The HD iPSC Consortium, 2012). Conversely, the authors found that in acute conditions, similar to the experimental design used in this project, where cells were dissociated and re-plated onto laminin substrate, no difference in NPC adhesion was observed. The results presented in this thesis contradict those results, as adhesion was found to be reduced in all PSC and striatal cell types in acute conditions. A possible explanation for this discrepancy could be due to the time given for cells to adhere; the results presented here were derived from a 15 min incubation so that the earliest changes in adhesion properties could be assessed, whereas the previous publication recorded levels of adhesion 12 h post-plating. It could be argued that these data show that mHTT is negatively regulating the initial attachment of cells to the culture substrate, however as this project did not conduct longer time-point analysis it is not possible to comment on the long term effect of mHTT on cellular adhesion.

3.6.2.3 Membrane properties of PSC-derived MSN-containing cultures exhibit subtle differences in maturity

The electrophysiological membrane properties of a neuron can give insights into its activity level, action potential generation ability as well as its maturity. It is possible to identify the presence of ion channels across the membrane, as well as discern how 'tight' or 'leaky' the
membrane is, whilst also estimating relative cell size. The data presented here suggest that there may be subtle differences dependent on mHTT, however as the control lines used in this project were not representative of each other, it is challenging to draw conclusions. In addition, the evidence in the literature on PSC-derived MSN membrane properties is also controversial. Xu et al (2017) found no differences in a range of membrane properties between control and mHTT-expressing lines, including membrane capacitance, input resistance and resting membrane potential (Xu et al., 2017). In contrast, the HD iPSC Consortium found that MSN clones expressing 180 CAG-repeats were unable to generate spontaneous action potential firing despite 60Q counterparts behaving in similar manner to controls (HD iPSC Consortium et al., 2012). Conforti et al identified a CAG-repeat length dependent decrease in single spike activity over time in MSNs expressing 60Q and 109Q (Conforti et al., 2018). Finally, Nekrasov et al assessed electrophysiology using a different technique - the authors quantified store operated calcium (SOC) entry in PSC-derived MSNs expressing mHTT and found that: SOC was increased in mHTT expressing lines; exon 1 was sufficient to produce the phenotype, as transient expression of exon 1 in control lines produced the same result; allele selective knock-down of mHTT suppressed the phenotype (Nekrasov et al., 2016). Whilst some variables such as MSN age/maturity, as well as assessment method may contribute to the conflicting results listed here, it is of paramount importance that an extensive investigation is carried out into the electrophysiological properties of PSC-derived MSNs; human neuronal activity cannot easily be studied on a single cell level in vivo, therefore without such a study, it will not be possible to conclude the impact of mHTT on MSN activity. The data presented by this project, have provided tentative evidence of membrane alterations in PSC-derived MSN-containing cultures, but also highlighted the importance of sample number and culture homogeneity; the cultures used in this project were heterogeneous in culture composition, and attempts to identify patched cells post-experiment were unsuccessful. Therefore future experiments would require positive cell identification prior to experimentation to ensure that it was indeed MSN membrane properties being assessed.

3.7 Limitations

Whilst every effort was made to ensure as pure a neuronal population as possible, it cannot be overlooked that full identification of culture composition was not achieved for PSCs subject to the MSN differentiation protocol. It was observed that cultures typically contained a proportion of both immature NPC-like cells and MSN-like cells within a broadly neuronal population. However, when quantified, these populations combined to less than 100%, thus the identity of some cells within cultures remains unknown. This has further connotations when interpreting phenotypical and functional differences between the cultures, as it cannot be guaranteed that the phenotype observed is due to the true MSNs or NPCs within the culture, or indeed an artefact or result driven by the ambiguous cells within cultures. This has been identified as a limitation of this thesis.
3.8 Summary

Adaptations were made to the Arber et al MSN differentiation protocol, that enabled reliable production of DARPP-32+/CTIP2+ neurons akin to MSNs in the human brain, including both direct and indirect pathway MSNs. Full characterisation revealed mHTT dependent effects on culture composition, including neuronal purity, proliferation rates and quantity of NSCs within cultures, some of which were maintained during ageing.

A variety of HD-associated phenotypes were observed in culture, suggesting that the HD mutation has an impact on cellular functioning in pre-pathology. Cell viability appeared compromised in mHTT expressing cells, however this was also affected by the age and maturity of the culture. Acute withdrawal of trophic support in the form of BDNF, rendered mHTT expressing cultures more vulnerable to cell death, however BDNF-deficient differentiation appeared to produce the opposite result, irrespective of genotype. Novel findings were shown regarding the effect of mHTT on cell adhesion, which was compromised in both NPC and MSN states; a continuation of compromised adhesion in mHTT expressing PSCs. Finally, only subtle differences were found in the membrane properties of cultures between genotypes, suggesting their electrophysiology is largely comparable, however there was a high degree of variability within experiments. In summary, the data presented here partially corroborate the results from HD PSC-derived MSN studies previously reported in the literature. This is the first known example of rigorous assessment in PSC-derived MSN-containing cultures from related individuals, complemented with isogenic lines. As such, the data presented here could suggest that genetic background and variability may impart a larger effect on cellular function than previously thought and should be accommodated in future studies.
Chapter 4 - Optimisation, characterisation and Huntington's disease phenotypes in iPSC-derived CPN-containing cultures

4.1 Background

It is well documented that HD is a multi-factorial disease and pathology develops throughout the entirety of the CNS. Whilst much investigation to date has focused on the most vulnerable cell-type in the disease, MSNs within the striatum, significant pathology and neurodegeneration occurs in other brain regions as a result of mHTT toxicity (Han et al., 2010). CPNs located in cortical layer V are also extremely vulnerable in HD, and evidence from both patient studies and mouse models has shown substantial neuron loss and dysfunction in this region during the course of HD (reviewed in Bunner and Rebec, 2016). Indeed, several studies have now identified a role for cortical pathology contributing to, or even driving striatal degeneration (Rosas et al., 2008; Thu et al., 2010), suggesting that this brain area may be of key significance in driving disease, thus more studies have now begun to focus on the contribution of cortical neurodegeneration to HD.

Most data on the effect of mHTT on the cortex has come from HD animal models, including knock-in, truncated and over-expression models (Cowan and Raymond, 2006; Raymond et al., 2011), as well as patient imaging studies and post-mortem analysis. Electrophysiological studies have suggested that cortical firing has a critical role in regulating the activity of the striatum via the CS pathway. The electrical properties of the axonal plasma membrane of cortical neurons are altered in R6/2 mice, with hyper-excitability evident early in disease progression (Burgold et al., 2019). Furthermore, energy (ATP) production and respiration has been found to be compromised in both PSC-derived neurons (The HD iPSC Consortium, 2012) and animal models of the disease (Han et al., 2010), which is thought to contribute to the hyper-excitable phenotype observed; a reduction in energy availability reduces the efficiency of the Na⁺/K⁺ ATPase, therefore the resting membrane potential of CPNs is more depolarised and the neurons are more easily excited (Estrada-Sánchez and Rebec, 2013). In theory, this leads to high levels of glutamate release in the striatum, which compounded with altered NMDA receptor expression on MSN dendrites, leads to excitotoxicity in the striatum (Kaufman et al., 2012). Furthermore, changes to synaptic density, as well as synaptic signalling are evident in HD CPNs. Burgold et al identified a significantly reduced density of synaptic proteins in cortical layers II/III in R6/2 compared to WT mice at eight weeks of age (Burgold et al., 2019). A reduction in inhibitory synaptic activity on layer V CPNs leads to large amplitude synaptic events occurring in the striatum (Raymond et al., 2011), which in combination with the excitotoxic priming events discussed in Chapter 3.1, could lead to excitotoxicity and degeneration.

The debate in the literature as to whether mHTT aggregates are a causative factor in HD pathogenesis is supported by a high aggregate burden in the cortex. In pre-symptomatic patients, Waldvogel et al identified a high percentage of neuronal intra-nuclear inclusions, as well as dystrophic neurites and neuropil threads, in the cerebral cortex, a finding that was not seen in the striatum. Furthermore, in juvenile HD cases, where motor symptoms more closely
resemble late-stage adult onset HD, a high HTT aggregate load has been identified in the cortex, which could indicate that aggregate burden correlates with symptom severity (Waldvogel et al., 2012).

Imaging studies and histological analysis of patient brains have also provided support for a more important cortical role than previously thought. Several groups have identified a correlation between atrophy of specific cortical regions and symptom presentation; patients who experience predominantly motor symptoms and little cognitive change present with primarily motor cortex atrophy (28% reduction), whereas those whose symptoms are dominated by cognitive changes present with limbic cingulate cortex atrophy (54% loss) (Rosas et al., 2008, 2002; Thu et al., 2010; Waldvogel et al., 2012), suggesting there is a clear-cut correlation between regional atrophy and functional phenotype. These are complemented by stereological cell counting evaluations demonstrating cortical atrophy in HD patient brains; multiple cortical areas showed significant cell loss including primary motor cortex (27% cell loss, predominantly motor symptoms) and cingulate cortex (34% cell loss, predominantly cognitive symptoms) (Thu et al., 2010). These findings are replicated to a certain extent by HD animal models, in which mHTT aggregate burden is high in the cortex (Ferrante, 2009), and both histological and imaging data recapitulate the cortical atrophy observed in HD patients (Bayram-Weston et al., 2012; Cheng et al., 2011; Zhang et al., 2010).

Similarly to MSNs, several human PSC-derived CPN differentiation protocols have been generated within the last decade (reviewed in Tousley and Kegel-Gleason, 2016). Arguably one of the most widely used and well characterised was devised by Shi et al and published in 2012. This protocol recapitulates the development of cortical neurons during embryogenesis and produces each layer of the cortex in a time-dependent manner; deeper layer neurons emerge first followed by the sequential emergence of more superficial layers (Shi et al., 2012). There have been numerous studies of PSC-derived MSNs, with regard to the impact of HTT polyQ-length on differentiation ability and efficiency, maturation rate and functionality (Conforti et al., 2018; HD iPSC Consortium, 2017; Mattis et al., 2015; The HD iPSC Consortium, 2012; Xu et al., 2017b). In contrast, the only study published to date that exclusively assesses the effect of mHTT on human PSC-derived CPNs was published by Mehta et al in 2018. The results proved interesting as it was found that PSC-derived CPN cultures expressing mHTT showed an altered transcriptomic profile. The DEGs were predominantly proteins regulating cell morphology and adhesion, the biological significance of which was corroborated by morphology and adhesion phenotypes observed in cultured CPNs. A second study, which compared the differentiation profile and maturation rate of MSNs and CPNs in both two-dimensions and three-dimensional cerebral organoids, was published by Conforti et al in 2017. This study showed that cerebral organoids expressing mHTT have disorganised cytoarchitecture, whereby clear separation of distinct cell types is not observed. When comparing the gross maturity of organoids to human brain samples, the transcriptomic profiles demonstrated that control organoids aligned most closely with inner and outer cortical plates, whereas HD organoids had a transcriptional profile similar to the ventricular zone/subventricular zone; mHTT expressing organoids had lower expression levels of cortical markers TBR1 and CTIP2, suggesting that the maturation rate of
cerebral organoids was impeded by the HD mutation (Conforti et al., 2018). Whilst informative, neither of these studies investigated any functional changes in neuron behaviour as a result of mHTT. Another study published recently, investigated the role of HTT in neurogenesis in PSC-derived CPNs and identified that once cells lose pluripotency, mHTT confers a loss of function phenotype on cellular division, leading to chromosomal instability and the appearance of giant, multinucleated neurons (Ruzo et al., 2018).

One key area of research into cortical pathology in HD involves BDNF production and processing. An essential function of CPNs within the CS pathway is to produce BDNF, which is then trafficked anterogradely along microtubules to axonal terminals within the striatum and released into the synaptic cleft in an activity-dependent manner (Kuczewski et al., 2009). There, it binds TrkB receptors on MSNs (Bayduyuk and Xu, 2014; Zuccato and Cattaneo, 2007), whereby the trophin-receptor complex is endocytosed and trafficked towards the cell body by retrograde transport (Lim et al., 2017). This neurotrophin is essential for MSN survival and function; BDNF signalling leads to the activation of three intracellular signalling cascades that mediate anti-apoptotic events, protein translation and synaptic plasticity (Bayduyuk and Xu, 2014). Deficiencies in BDNF processing by CPNs may therefore contribute to, or even cause, striatal degeneration. Indeed, several groups have found mHTT-dependent phenotypes in BDNF production, trafficking and release in HD animal models, cell lines and human tissue (Bathina and Das, 2015; Ferrer et al., 2000; Zuccato et al., 2009). As of yet, it is not known if these phenotypes are replicated in human neurons in vitro i.e. in PSC-derived CPNs. As PSC-derived neurons have not been found to contain mHTT aggregates, it is important to determine whether BDNF deficiencies are present at the pre-pathological stage, thus PSC models might enable study of very early pre-pathological changes in neuronal function in the most physiologically relevant human cell model currently available.

4.2 Aims

1. Optimise a protocol for PSC differentiation into layer V CPNs.
2. Characterise the full cohort of PSC-derived CPN-containing cultures with HTT polyQ-lengths in the pathogenic and non-pathogenic range, for cell identity and culture composition.
3. Generate novel methods to study mHTT-dependent phenotypes emerging in CPNs in culture.
4. Assess CPN morphology and axonal dynamics.
5. Assess aspects of BDNF production, trafficking and release in CPNs.

4.3 Methods

A summary of methods used in this Chapter is outlined below. Full details of experimental protocols can be found in Chapter 2: Materials and Methods.

Pluripotent stem cells from both the HD family series and IsoHD were cultured as described in section 2.2, prior to differentiation into layer V CPNs using an adapted version of the Shi et al
protocol (section 2.4). The adaptations are described in sections 2.4.2 and 2.4.4 and expanded upon here.

The first adaptation pertained to the cortical passage procedures. Following on from the success of the EDTA passage of PSC-derived MSNs, CPN cultures were subject to EDTA passage as described in Arber et al (2015). These were completed on the days in which dispase splits would have originally taken place i.e. d12 and d22. Using the EDTA passage procedure, culture viability and consequently differentiation success was consistently increased; no overt morphological differences or changes to culture composition were observed in terminal cultures as a result of the change in passage protocol.

Whilst the EDTA passage was successful in increasing the culture viability and differentiation success, it was noted that a population of proliferating cells persisted and often dominated some cultures. The extent of this varied between differentiations of the same clone, and also between clones, therefore was challenging to control for in terms of culture composition. In an attempt to reduce the numbers of proliferating cells, an extra Accutase passage was trialled between d40-60. Differentiating cultures were robust enough to survive the additional passage and it was observed that this addition could reduce the numbers of proliferating cells within cultures – as assessed by ki-67 immunofluorescence – as well as improve plating distribution enabling easier experimentation. As this passage was completed at a time point in which layer V and VI neurons were at peak emergence, there were concerns this could reduce the percentage of deep layer neurons in terminal cultures. However, upon image analysis, it was evident that a high percentage of deep layer neurons persisted in culture, despite the additional late-stage passage. Furthermore, a higher neuronal purity was achieved in cultures subject to the additional passage. However, as this passage was completed at such a late time point, it could only be employed in cultures that were designed to age past 70 days in culture.

In cultures where a high percentage of proliferating NSCs remained evident, a dosing period of the γ-secretase inhibitor DAPT was trialled. DAPT down-regulates the Notch signalling pathway and encourages cells to exit the cell cycle, thus is termed a pro-differentiation agent (Boissart et al., 2013). Personal communications from Dr Selina Wray lead to the design of an experiment in which persistently proliferating cells of both 22Q and 75Q genotype cultures were subject to 10 µM DAPT treatment every 48 h for 7 days, commencing at d25. At the termination of dosing, cultures were observed and scored for ‘neuronal morphology’. It was observed that DAPT treatment increased the proportion of cells in the culture that fulfilled a neuronal phenotype criteria (polarised cells, neural processes evident, multi-neuritic, small somal size). Furthermore, this phenotype appeared to persist for several days after the termination of treatment, so much so that the neuronal phenotype was maintained until d50 whereby cells could be used for further experimentation.

Characterisation of CPN-containing cultures was carried out using phase contrast microscopy and confocal microscopy, detailed methods of which are in section 2.5. For image capture
completed with the high content screening platform, adjustments were made to the culture protocol as detailed in sections 2.5.2, and 2.5.3. Analysis was carried out using Columbus cloud-based software (section 2.5.3.1). To assess the emergence of HD phenotypes, a variety of methods were used. Morphological analysis of CPN cultures was carried out using an IncuCyte longitudinal imaging platform (section 2.5.5) and in-built analysis software was used for quantification. An adhesion assay previously published in the literature was used for this project (Casey et al., 2015), however optimisation was carried out prior to experimentation (section 2.6.1). The viability of CPN-containing cultures at d50 was assessed by running cell supernatant samples on a commercially available lactate dehydrogenase assay (section 2.6.1); absorbance values were normalised to internal controls for each experiment. Total protein concentration of cell lysates was quantified using the Pierce BCA protein assay (section 2.6.2). The concentrations of BDNF released into cell supernatant and stored intracellularly were calculated by running CPN conditioned media samples on a commercially available BDNF ELISA kit (section 2.7.3). Samples were either collected in baseline conditions or after treatment with a stimulatory agent (section 2.7.2).

Microfluidic chambers were constructed as described in Park et al (2006) (section 2.8). Specifically, production of the MFC PDMS insert was completed using templates supplied by Prof Giampietro Schiavo, under the supervision of lab members (section 2.8.1) prior to transport to the Tabrizi lab wherein sections 2.8.2, 2.8.3 and 2.8.4 were completed. To assess the axonal cytoskeletal dynamics of cell cohorts, CPNs were seeded into fully constructed and prepared MFCs and maintained as described (section 2.8.4). Several pilot experiments were completed to identify the optimal conditions for axonal crossing experiments (sections 2.9.2.1) prior to running the full experiment as described in section 2.9.2.2.

Statistical analysis was completed as described in section 2.11.

4.4 Contributions

- Dr Nicole Birsa and Dr Ione Meyer generated and optimised the original MFC templates used to reproduce MFC inserts.
- Matthew Bentham provided the optimised protocol for MFC cell seeding.

4.5 Results

4.5.1 Generation of PSC-derived CPN-containing cultures
The HD family series and IsoHD series were differentiated to layer V CPNs over the course of 50 days using an adapted version of the Shi et al differentiation protocol (2012).

4.5.1.1 Modification of the Shi et al protocol
The original Shi et al protocol was successful in generating layer V neurons, but was unreliable. The cause of this inconsistency was due to the Dispase splits which were designed to increase the purity of neurons in the culture. This procedure is very harsh on the cells as it requires a minimum of 10 min incubation with Dispase, followed by at least three washes which are
designed to break up the cells. A high proportion of splits resulted in whole culture death either due to cell death during the passage, or an inability of viable cells to attach to the culture substrate post-passage, resulting in culture death. As such, the protocol was adapted in two ways to compensate for Dispase split removal.

4.5.1.2 Neural stem cell generation

After 12 days of dual SMAD inhibition as per the method in Shi et al., 2012, morphological changes were evident in differentiating CPN cultures (Figure 4. 1). The nuclear to cytoplasmic ratio had decreased, NSCs were easily identified due to their classic paving-stone morphology, and NSCs could be observed clustering into pre-neural rosettes. Observationally, NSCs gradually darkened in colour and began showing signs of polarisation. This became more evident after the second passage completed at d22, whereby within 24 h post-passage, neural rosette structures (Figure 4. 2A) were evident. PSC genotype did not appear to alter the ability of cultures to form neural rosette structures. Depending on the density of cultures, neural processes were evident from ~d25 onward. To confirm the presence of NSCs within differentiating cultures, culture composition was analysed by confocal microscopy probing for NSC marker nestin, and proliferating cell marker ki-67 (Figure 4. 2B).
Figure 4. Phase contrast images of PSC-derived CPN-containing cultures showed neural progenitor cells forming post-passage 1. Using a brightfield microscope on 10x magnification, differentiating CPNs were imaged within three days post-passage 1 (EDTA protocol). Representative phase contrast images demonstrate the reduced nuclear to cytoplasmic ratio, supported by the increase in phase contrast, indicating the cells were losing their pluripotency phenotype. No morphological differences were observed between cultures of HD family 22Q and 75Q genotype of any clone (1-3) assessed. Scale bars represent 50 μm.
Neural rosette formation and positive identification of NSCs within differentiating cultures. After completion of the second passage, neural rosettes were evident in HD family series cultures irrespective of genotypes (A). The presence of NSCs and proliferating cells within these rosettes was confirmed with immunofluorescence staining (B) of NSC marker, nestin (white) and proliferating cell marker, ki-67 (green) for both genotypes. Scale bars represents 100 μm (A), and 50 μm (B).

4.5.1.3 Cortical layer V generation

The Shi et al protocol (2012) generates the six distinct layers of the cortex in an inside-out manner, meaning that the deeper layers (V and VI) emerge first, prior to the more superficial layers emerging in a time-dependent manner. As this project was focused on recapitulating the CS pathway, cultures were usually maintained until d60 when the key target of interest, layer V, was expected to have emerged. To confirm that the neurons being produced through differentiation were indeed akin to layer V CPNs in the human brain, a series of immunofluorescence experiments were designed to fully characterise the cells.

Representative images of both 75Q and 22Q cultures can be seen in Figure 4. 3A and B respectively. The confocal images depict a high proportion of cells expressing both the layer VI marker TBR1 and the layer V marker CTIP2, as well as demonstrating a high neuronal purity (NeuN) despite less than optimal far-red signal strength and a weak antibody. To quantify the
exact percentage of neurons in culture that were positive for the marker of interest (CTIP2), HCI was used to collect a large, unbiased image database that could be subject to unbiased automated analysis. Initially, the percentage of viable cells was calculated (Figure 4.4A), here a significant reduction in viability was found between HTT 22Q and 75Q cultures. The mean viability of 22Q cultures was 95.93%, conversely 75Q cultures reached only 90.7% survival ($p = 0.0001$). Subsequent analysis was calculated as a percentage of viable cells only, therefore condensed or necrotic nuclei that co-stained with the protein of interest were excluded from analyses. The proportion of CTIP2 positive cells did not differ between genotype (22Q = 27.77%, 75Q = 29.02%; $p = 0.823$) (Figure 4.4B). Similarly, there was no significant difference in the proportion of proliferating cells (ki-67+) within cultures (Figure 4.4C), (22Q = 24.23%, 75Q = 27.88%; $p = 0.675$). However, when assessing the proportion of cells within cultures that were positive for the NSC marker, nestin, a significant difference was found (Figure 4.4D). Cultures expressing the HD mutation comprised nearly double the percentage of neural stem cells than 22Q cultures (14.73% vs 28.42%; $p = 0.0043$). As the percentage of layer V neurons, and proliferating cells did not appear to differ, it was concluded that the differences in culture composition percentages were derived from other cell types within the culture; this protocol is known to generate glial cells over time, as well as the other layers of the cortex of course.

4.5.1.4 Upper layer neuron differentiation

Although this project only called for the production of cortical layer V, it was important to ascertain whether the PSCs used were able to complete the full differentiation protocol and successfully differentiate into the upper layers of the cortex. SATB2 is a cell marker that is common to all upper layers (I-III) (Shi et al., 2012), therefore cultures that had been aged to 90 days were fixed and probed for SATB2 in combination with the layer V marker, CTIP2. It was identified that both 75Q and 22Q cultures produced a small proportion of SATB2 positive cells at this time point in culture (Figure 4.3C and D). As the differentiation protocol develops in an inside-out, time dependent manner, it is plausible that these positively identified cells represent the emergence of layers IV and III, as the most superficial layers are not predicted to emerge until over 100 days in culture.

4.5.1.5 Non layer-specific cortical neuron markers

Whilst it was clear that the differentiations completed during this project were indeed producing the correct cortical neurons, a secondary level of characterisation was carried out assessing the expression of pan-cortical markers. One of the key proteins that was assessed was BDNF and immunofluorescent staining was completed by confocal microscopy on 22Q and 75Q cultures, as depicted in Figure 4.3E. The representative images clearly display the ‘firework’ phenotype typical of BDNF staining; no overt difference in expression level was noted between genotypes. Furthermore, BDNF co-localised with both the pre-synaptic marker, SYT1, as well as pan-neuronal marker, βIII-tubulin, which positively stains neurites.

The majority of CPNs in the brain are excitatory and thus produce glutamate as a neurotransmitter and express its associated markers. As a further level of characterisation, PSC-derived CPNs were probed for vesicular glutamate transporter 1 (vGLUT1) using confocal
imaging. As can be observed in Figure 4. 3G (left panel) at 40x magnification, there is clear co-staining of vGLUT1 with the neuronal marker, βIII-tubulin. To ensure that the signal observed was specific, a high magnification zoom was applied to the area within the red highlighted box. Images taken on high magnification were split by channel (right panels) and clearly show positively stained vesicular puncta, which align with βIII-tubulin fibres.

Figure 4. 3. Representative panel of confocal images showing expression of key cortical neuron markers in PSC-derived CPN-containing cultures. Immunofluorescence images were captured by confocal microscopy. Cultures were probed for a variety of pan-cortical and layer specific markers to
ensure cultures had successfully completed differentiation and could be classified as cortical neurons. Cortical layers V and VI are identified by positive staining of CTIP2 and TBR1 respectively, whilst βIII-tubulin is a pan-neuronal marker. Representative images of 75Q cultures (A) and 22Q cultures (B) at d50. Upper layers of the cortex emerge at later time points in culture; SATB2 is a layer III-I marker and can be identified in both 75Q (C) and 22Q (D) neurons after d90. Cortical neurons produce and store BDNF intracellularly; production of this neurotrophic factor was confirmed in both 75Q and 22Q cultures (E). The presence of vGLUT1 was confirmed (F) with a high magnification zoom of the red rectangle area identifying punctate vesicles. Scale bars represent 50 μm.

Figure 4. Quantitative analysis of culture viability and composition using HCl immunofluorescence. The images captured by HCl were subject to automated script-based analysis. Using Columbus software, analysis scripts were designed unique to each antibody pairing (well set) and once optimised, applied to all clones that had been probed with that well set. Genotypes were pooled to generate, for day 60 cultures, an average percentage of viable cells in the culture (A), percentage of layer V neurons as indicated by CTIP2 staining (B), the percentage of proliferating cells as identified by ki-67 staining (C) and the proportion of NSCs in the culture, calculated by nestin staining (D). All percentages are calculated as a percentage of viable cells. Data are presented as mean ± SEM. (N = 1, n = 2, n ≥ 12). ** p < 0.01, *** p < 0.001.

4.5.1.6 Synaptic markers

To assess the expression and localisation of synaptic proteins in PSC-derived CPN-containing cultures, triple immunolabelling experiments were carried out, probing for the pre-synaptic marker, synaptophysin, the post-synaptic density protein, PSD95, and MAP2, which is highly expressed within dendritic processes. As can be seen in Figure 4. 5A and B respectively, both 22Q and 75Q cultures show robust expression of synaptic markers. Moreover, the post-synaptic marker, PSD95, is highly co-localised to MAP2 positive processes, with synaptophysin being less so, indicating that the localisation of both proteins appears to be correct. Unfortunately the magnification with which these images were taken was not high enough to resolve individual synapses, however, in an attempt to quantify the levels of synaptic juxtaposition, a script was written using Columbus software, which aims to identify the proportion of pre-synaptic signal that is co-localised to post-synaptic signal within a MAP2 positive neuron. The resulting analysis can be observed in Figure 4. 6. The total puncta counts of pre-synaptic proteins when normalised to viable MAP2+ cells was significantly different between genotypes. Control cultures showed significantly reduced levels to both 58Q and 75Q cultures (p = 0.019, p = 0.0056) (Figure 4. 6A). The numbers of normalised post-synaptic puncta did not differ between genotypes (control vs 58Q, p = 0.54; control vs 75Q, p = 0.81) (Figure 4. 6B). Secondary analysis included identifying the number of MAP2 positive neurons in the cultures that contained co-localised pre- and post-synaptic puncta calculated as a percentage of MAP2+ neurons overall (Figure 4. 6C); however, no significant differences were found in this analysis.
Representative images of pre- and post-synaptic markers in PSC-derived CPN-containing cultures at d60. HCI and analysis was performed to identify the presence of synapses within cortical cultures. The pre-synaptic marker synaptophysin, and post-synaptic marker PSD95, were probed for alongside dendritic marker MAP2. Cortical cultures of both 22Q (A) and 75Q (B) genotype were found positive for both markers, in a typical expression pattern. Scale bars represent 50 μm.

Quantification of synaptic markers in PSC-derived CPN-containing cultures from HCI analysis. Images captured on the high content screening platform of pre- and post-synaptic proteins synaptophysin and PSD95, were subject to automated script analysis. Individual puncta were isolated and the sum of puncta per replicate was calculated and normalised to viable MAP2+ cells for pre-synaptic protein (A) and post-synaptic protein (B). To identify the localisation of puncta, the number of viable MAP2+ cells that co-stained with both pre- and post-synaptic puncta were quantified and calculated as a percentage of the total MAP2 positively stained cells (C). The data obtained from 22Q and 30Q genotypes was combined as control. Data are presented as mean ± SEM (N = 1-2, n = 2, n ≥ 12) . **, p < 0.01.

Due to the wide range of HTT polyQ-lengths available for study during this project, it was possible to investigate not only if mHTT-dependent phenotypes were arising in vitro within a relatively short time period, but also if these phenotypes occurred in a polyQ-length dependent manner. Therefore, once cortical characterisation was complete, and there was confidence in the reliability of differentiations, investigation began into identifying any functional differences between genotypes, and the mechanisms behind them.
4.5.2 Cell viability

To identify whether expression of mHTT impacted culture viability, a series of experiments were conducted in which the LDH levels released from CPN-containing cultures at d50 were assessed. The concentration of LDH release was assessed in baseline conditions, i.e. with no stimulation, stress or treatment and normalised to total cell number (total LDH release). The data shown in Figure 4. 7A demonstrates that viability does differ between genotypes, and in some cases, between clones of the same genotype [clone 22Q (2) significantly reduced compared to clones (1; p = 0.0007) and (3; p = 0.0592)]. By combining the data from multiple clones of the same genotype however, it was possible to better gauge if mHTT per se impacts viability. As seen in Figure 4. 7B, a significant reduction in viability (3.21%) was observed between 75Q clones when compared to control (22Q and 30Q data combined) (p < 0.0001) [average viability control = 91.01%, 75Q = 87.8%].

4.5.3 Cellular adhesion

As neuronal morphology appeared to differ between genotypes, further investigation was warranted into the functional properties of the PSC-derived CPN-containing cultures. It had been noted during the course of this project that the levels of adhesion appeared to differ between cultures. Particularly during early stages of differentiation, such as between passage one and two, HD cultures appeared to have reduced adhesion compared to control cultures - this was observed in both HD family series and the IsoHD series. For this reason, a series of adhesion assays were designed to test the adherent properties of the cells at different time points in culture. As discussed previously (section 3.5.3.1), PSCs expressing mHTT demonstrated reduced adhesion to Geltrex than their 22Q or 30Q counterparts (22Q vs 75Q p < 0.0001; 30Q vs 45Q p < 0.0001; 30Q vs 81Q; p < 0.0001). The adhesion of NPCs to a laminin substrate was tested at d30 in differentiating CPN cultures. The data obtained from 22Q and 30Q cultures was combined and termed control, and was found to have significantly increased
adhesion compared to 75Q cultures (p <0.0001) (Figure 4. 8B). Specifically, it was observed that 75Q NPCs had on average a mean difference of 84.45 adhered cells when compared with control. Examples of this are shown in Figure 4. 8E. To identify whether the reduced adhesion phenotype persisted into maturity, the adhesion assay was repeated on cultures aged to 50 days (Figure 4. 8C and F). Interestingly this was not the case; 75Q neurons adhered equally well to the substrate as control neurons (p = 0.9123).

Figure 4. 8. Quantification and representative images of adhesion assays on PSCs, cortical NPCs and mature CPNs. The adhesion ability of PSCs and PSC-derived neurons at different stages of differentiation was assessed using an in-house adhesion assay. A) PSCs expressing mHTT from both the HD family series (75Q) and IsoHD series (45Q, 81Q) had reduced adhesion compared to control counterparts (22Q, 30Q). B) Cortical NPCs assessed at d30 demonstrated reduced adhesion in the 75Q line compared to control NPCs. C) No difference was observed between genotypes of CPN-containing cultures assessed at d50. Representative images of cultures post-adhesion assay for D) PSCs, E) NPCs and F) CPNs. Three FOV were manually quantified for each clone which was run in duplicate. Data are presented as mean ± SEM; (N =1-2, n = 2, n ≥ 16) **** p < 0.0001.

4.5.4 Neuritogenesis and culture topology
During the course of CPN differentiation it became evident that during ageing, the neurons behaved differently, in particular with regard to their neurite morphology. Whilst plating and culture conditions remained constant between clones, at later stages of differentiation the mHTT expressing clones showed a more bipolar morphology, with shorter processes that formed a sparser network across the culture dish. Using the IncuCyte longitudinal imaging platform, with
in-built NeuroTrack software, CPN-containing cultures of either 22Q or 75Q genotype of the HD family series were then analysed for differences in neurite outgrowth and cell cluster number and area. NeuroTrack software segments the culture area imaged into either ‘Cell clusters’ or ‘Neurites’ (see Figure 2.5). Data collected includes average neurite length, number of branch points, number of cell-body clusters, and cell-body cluster area (Figure 4. 9A, C, E, G). Neurite length and branch point number can also be normalised to cell-body cluster metrics, giving an average neurite length/branch point per cell cluster (Figure 4. 9I & K). As the initial measurements of each of these parameters tended to differ between genotype e.g. 22Q neurite length started at 31.39mm, whereas 75Q started at 20.51mm, an overall percentage rate of change was calculated to accommodate these discrepancies (Figure 4. 9B, D, F, H, J, L, N, P). To analyse the data, a repeated measures ANOVA with Bonferroni correction was calculated, as well as linear regression analysis (Table 9). For the overall percentage change, a Student's t-test was used to compared genotypes.

4.5.4.1 Neurite length

Overall, the average neurite length appeared longer in 22Q cultures compared to 75Q cultures at each time point (Figure 4. 9A & B). Within a repeated measures ANOVA of the data, the HTT polyQ-length accounted for 38.02% of the variation (p < 0.0001), however this result only reached significance at time points 60 h and 78 h when Bonferroni corrected for multiple comparisons (p = 0.48, p = 0.0264). Similarly, the overall percentage change did not significantly differ between groups (p = 0.6512). Linear regression analysis of the slopes returned a trend towards significance (p = 0.056).

4.5.4.2 Branch points

When assessing the number of branch points between groups, there was a significant interaction between time and polyQ repeat length, accounting for 6.407% of the variation (p = 0.0147). Both time variable, and polyQ length also reached significance, accounting for 21.9% and 36.91% of variation respectively (p < 0.0001). When adjusted for multiple comparisons, no significance was found between genotype at the initial time point (p = 0.403), however all subsequent time points were significant. Accounting for the discrepancy in starting value, when assessing the overall percentage change, there was a trend towards reduced numbers of branch points in 75Q cultures compared to 22Q (p = 0.063) (Figure 4. 9C & D). Linear regression analysis of the slopes showed no significant difference between genotypes (p = 0.842).

4.5.4.3 Cell-body clusters

By assessing the number of cell-body clusters in a culture over time, the proliferation rate of cells could be assessed. Previous data (percentage of ki-67+ cells in culture) had suggested that 22Q cultures had a higher proportion of persistent proliferating cells. It was found that time and HTT polyQ-length contributed 37.41%, and 20.19% variation overall (p < 0.0001), however, only 78 h returned a significant difference when assessing time points individually, after correction for multiple comparison (p = 0.0452). There was no significant difference found in the
overall percentage change (p = 0.2023) (Figure 4. 9E & F) and linear regression analysis did not find a significant difference between slopes.

4.5.4.4 Cell-body cluster area

By determining the total area covered by cell-body clusters compared to neurites, it is possible to discern any differences in cell body size or clustering behaviour. This result proved interesting, as a significant interaction was observed between time and HTT polyQ- length (10.30% variation, p < 0.0001), plus both time variable and polyQ-length accounted for significant percentages of variation (35.98%, 39.34% respectively, p < 0.0001). Furthermore, when assessing each time point, a significant difference was observed in all but the initial measurement (p < 0.012 - 0.0001). Similarly, the rate of change was significantly increased in 22Q cultures compared to 75Q cultures (p = 0.0012), suggesting that 22Q cultures have increased clustering tendency or proliferate more, leading to an increased somal area over time (Figure 4. 9G & H). Finally, linear regression analysis showed a highly significant difference between slopes (p < 0.0001), so much so that the intercepts could not be compared.

4.5.4.5 Neurite length per cell cluster

When assessing neurite length per cell-body cluster, is was possible to get an approximation of the neurite length per cell. It is important to note, however, that the NeuroTrack software is limited in its thresholding, therefore a ‘cell-body cluster’ frequently accounted for more than one cell-body. As such, the values generated for neurite length per cell are very small. It would be possible to calculate the average neurite length per cell by manually counting the number of cells per image and calculating the overall neurite length as a function of this. However, as each data point is an accumulated average from multiple independent experiments, in which six replicates sampled four FOV per time point, this process would be too time consuming to complete.

As can be seen in Figure 4. 9I & J, there was no difference between either the average neurite length per cell-body cluster (p > 0.99), or the overall percentage change between 22Q and 75Q cultures (p > 0.1562), and linear regression returned a non-significant result (p = 0.419). In fact, the only significant result was that of the time variable contributing 29.66% of variation (p < 0.0001).

4.5.4.6 Branch point per cell-cluster

The number of branch points could be analysed as a function of number of cell-body clusters. When calculated as a function of cell-body clusters, there was a significant difference in both time and polyQ-length variables contributing to 8.399% and 49.6% variation respectively (p = 0.0043, p < 0.0001), but only time points 110 h and 114 h survived correction for multiple comparisons (p = 0.0359, p = 0.0166) (Figure 4. 9K). The overall percentage change in number of branch points per cell-body cluster did reach significance, as shown in Figure 4. 9L (p = 0.0394) and regression analysis returned a highly significant difference in the slopes (p < 0.0001).
Morphological differences are found between 22Q and 75Q CPN-containing cultures when assessed longitudinally. Using an IncuCyte longitudinal imaging platform, several parameters of neuronal morphology were assessed in cultures at 50 days’ differentiation. The average neurite length (A), number of branch points (C), number of cell-body clusters (E) and cell-body cluster area (G) were quantified across 24 images per time point per experiment for 22Q and 75Q neurons. The overall percentage change for each of these measures was calculated (B, D, F, H). To calculate per cell values, neurite length (I) was calculated as a function of cell-cluster number; percentage change was calculated in the same manner as described (J). The same process was completed for branch point number (K, L). Data points represent mean ± SEM, 24 FOV per time point, per clone (N = 1, n = 2, n = 16). A repeated measures ANOVA was completed on time course data; a Student’s t-test was completed on rate of change data. Linear regression analysis was completed on the data slopes * p < 0.05; ** p < 0.01; **** p < 0.0001.

Table 9. Summary of Bonferroni post-hoc multiple correction of IncuCyte data, and corresponding linear regression analysis.

<table>
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<tr>
<th>RM ANOVA (Bonferroni)</th>
<th>Time point (h)</th>
<th>Neurite length p value</th>
<th>Branch points p value</th>
<th>Cell-clusters p value</th>
<th>Cell-cluster area p value</th>
<th>Neurite length/cell-cluster p value</th>
<th>Branch point/ cell-cluster p value</th>
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</table>

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4.5.5 Axon dynamics

Due to the previous findings in which both morphology and cellular adhesion were found to differ in HD CPN-containing cultures, attention was focused on delving deeper into the mechanism by which this could be occurring. It was hypothesised that the actin cytoskeletal dynamics, in terms of polymerisation and depolymerisation, may be deficient in HD CPNs. As a proxy readout for actin cytoskeletal dynamics, the rate of axonal projection was assessed through MFC micro-channels. The rationale behind this was, if axonal dynamics were compromised a slower projection rate would be expected and vice versa. Identifying whether the HTT polyQ-length impacts cytoskeletal dynamics had important connotations within the context of this thesis and the field of HD as a whole, because if proven correct such that actin cytoskeleton modelling and remodelling occurred at a slower rate or is deficient in any other way in CPNs carrying the HD mutation, there is an argument to be had as to the degree to which the CS pathway forms correctly in the brain of HD gene carriers. This project was designed to investigate whether there was an impairment in the initial CS connections formed during development, however this is just one of many possible reasons as to why CS pathway connectivity might be impaired in HD.

4.5.5.1 Axon crossing pilot experiment

A pilot experiment was designed to identify if PSC-derived CPN-containing cultures were able to survive and project through micro-channels in MFCs, aiming to compare four measures of axonal dynamics between two 22Q clones and one 75Q clone. MFCs are incredibly useful devices that provide fluidic separation between cell chambers whilst allowing physical cell interaction. The early interactions between CPNs and MSNs has yet to be studied in a human model in vitro; by using MFCs, these experiments could potentially recapitulate early events in the establishment of CS pathways in vivo. For reference, the table of measures assessed as presented in Chapter 2 (Table 6) has been included here (Table 10). The first measure aimed to identify how exploratory the neurons were in terms of projection and retraction of neural processes. This measure was assessed by counting the number of neuritic projections that had entered the micro-channel openings every 48 h for seven days. The second measure was aimed at assessing axonal projection ability, and comprised daily quantification of the number of axons emerging into the axonal chamber of MFCs. The third measure assessed the overall number of projected axons at the termination of the study, and as such the projection efficiency could be calculated by dividing the number of successfully projected axons, by the total number that had entered the micro-channel openings. Finally, the fourth measure was an analysis of the axonal networks that had been formed by cortical axons post-projection. This measure was based on the scoring system described in 2.9.2.1, and was aimed at identifying the levels of interaction between cortical axons.

This pilot experiment demonstrated that PSC-derived CPN-containing cultures could survive in and successfully project across the micro-channels within the MFCs, meaning this in vitro system could be utilised to measure key phenotypes in early CPN axonal projection. The pilot
data also suggested that 75Q cultures were superior in their pathfinding ability to 22Q clones as demonstrated by a higher channel occupancy across the length of the experiment (d7 22Q = 76 vs 75Q = 136) however, when the percentage change was calculated, by normalising d7 to d1 values, it was apparent that this was not correct (Figure 4. 10A), although the rates of each control clone were not significantly different. Despite subtle differences in the number of axons entering the micro-channels, all of the clones struggled to successfully project their axons the whole way through to the axonal chamber over the experimental period; no significant difference was observed in the rates of projection between 22Q and 75Q clones (Figure 4. 10B). One contributing aspect to this was the variability in MFC construction itself; it was noted that particularly in the MFCs blindly allocated to control clones, some of the micro-channels remained blocked by coating substrate (laminin), which hindered axonal projection through micro-channels, therefore it was noted that further optimisation would be required in order to accurately assess CPN axonal projection. The success rates of the clones in terms of total number of projected axons also did not differ and the projection efficiency [100 x (Measure 3/Measure 1)] was also similar between clones (22Q = 11.14%, 75Q = 7.82%) (Figure 4. 10C). Unfortunately, this experiment had to be terminated at d7 as the optical clarity of the devices was compromised and the final measure of network complexity could not be assessed. Furthermore, although the variance of measure one was extremely small, the variability of measures two and three meant that no clear conclusions could be drawn. It was clear that further optimisation was required to increase the efficiency of cortical axon crossing within the MFC devices.

Figure 4. 10. Axon crossing pilot experiment. A pilot experiment was designed to identify if PSC-derived CPN-containing cultures were able to survive and project through micro-channels in MFCs. The experiment set out four measures to be assessed over the period of 10 days, targeting different aspects of neuronal functionality. A) The number of cells projecting neural processes into micro-channel openings was assessed over a period of 7 days [measure 1]. The rate of channel occupancy was calculated by dividing the last time-point value by the first. B) The number of axons projecting through micro-channels into the axonal chamber was quantified every 24 h for 7 days [measure 2]. C) The percentage axon crossing efficiency was calculated by dividing the d7 values of measure 2/measure 1. Data are presented as mean ± SEM; n = 3.
<table>
<thead>
<tr>
<th>Biological process</th>
<th>Measure</th>
<th>Description</th>
<th>Days assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>How exploratory are the axons?</strong></td>
<td>5. Channel occupancy</td>
<td>The number of micro-channels (total 168/MFC) occupied by 1 or more neural projection.</td>
<td>1, 3, 5, 7</td>
</tr>
<tr>
<td><strong>How fast are the axon cytoskeletal dynamics?</strong></td>
<td>6. Latency to cross</td>
<td>The number of axons successfully projected through to the axonal compartment.</td>
<td>Every 24 h</td>
</tr>
<tr>
<td><strong>How efficient is the culture in terms of projection?</strong></td>
<td>7. Projection efficiency</td>
<td>The total number of axons projected through to the axonal chamber divided by the total number entered.</td>
<td>End of experiment (d7)</td>
</tr>
<tr>
<td><strong>Are the neurons capable of generating an axonal network?</strong></td>
<td>8. Network complexity</td>
<td>An assessment of how complex the projected axonal networks are: axonal interactions and branching points, evidence of synaptic swellings. Scoring system as follows: 0 = no networks formed/no interactions. 1 = axons are in close proximity to each other. 2 = axons look to be interacting with each other in a simple network. 3 = axons are interacting with each other in a complex network - multiple interaction points on same axons and multiple axon interactions.</td>
<td>End of experiment (d7)</td>
</tr>
</tbody>
</table>

### 4.5.5.2 Axon crossing stimulation pilot experiment

Several avenues of study were investigated to increase the efficiency of cortical axonal projections through MFC devices in order to facilitate the study of early CS-pathway development. Although shortening the length of the micro-channels would have almost certainly have increased axonal crossing, this was not possible due to the lack of appropriate MFC templates, therefore a biological solution was investigated.

Both GDNF and nerve growth factor (NGF) are key chemoattractant molecules for CPNs (Drescher, 2011; Markus et al., 2002). Furthermore, as CPNs project their axons towards the striatum in vivo, it was hypothesised that the MSNs themselves may be secreting trophic or chemoattractant factors which would stimulate projection. To assess if the above factors could influence the projection rates of cultures, a single HD clone [75Q (3)] was plated into the somal
chamber of MFCs and subject to the above treatments, all of which were compared to baseline conditions (unconditioned N2B27 media, referred to as control). To assess if GDNF and NGF had a dose dependent effect, three doses were trialled. To assess if the trophic secretions of MSNs differed according to whether they expressed mHTT or not, conditioned media from 22Q and 75Q MSNs aged to d36 was collected. The experimental design from section 4.5.4.1 was replicated and the investigation was scheduled to run for ten days.

As can be seen from Figure 4.11, neither GDNF (A), NGF (B) or MSN conditioned media (C) had a stimulatory effect on channel occupancy (measure 1) above control levels. However, when assessing the second measure (latency to cross), although GDNF had no effect at any dose (Figure 4.11D) it was found that NGF was able to dose-dependently increase the number of axons successfully projecting into the axonal chamber above that of control levels [d10 50 ng/mL = 14, 100 ng/mL = 23, 200 ng/mL = 29] (Figure 4.11E). Furthermore, whilst 22Q MSN conditioned media also had no effect above control [22Q MSN = 11.5 vs control = 9], the data presented in Figure 4.11E shows that media conditioned by 75Q MSNs had a detrimental effect on cortical neuron projection [75Q MSN = 1.5 vs control = 9], however further investigation would be required to confirm this result in other lines. These experiments were not completed within this project as they were not deemed essential to the study; this was a pilot experiment designed to explore if chemical stimulation could increase axonal crossing with a view of optimising conditions for CS co-culture.

As these experiments were completed in a line expressing mHTT, it was important to establish whether the effects seen were specific to HD cultures, or if control cultures would also behave in the same manner. As no effect was seen with GDNF in the previous experiment, this was not tested again. Firstly, assessing measure 1 in 22Q cultures, again treatment did not appear to impact the number of channels occupied by neural processes (Figure 4.11J). There was an initial decrease percentage of occupancy (~80% d1) in MFCs treated with 22Q MSN-conditioned media, which was reduced over time (6% d7). When assessing projection latency (measure 2), it was found that that although 200 ng/mL NGF was able to substantially increase axon crossing above other treatment groups (~270% above most other treatments), this was still deficient when compared to control media alone (Figure 4.11K). Indeed, when comparing 22Q and 75Q cultures treated with control media alone, a striking difference was observed (Figure 4.11O). In control conditions, 22Q cells were able to project axons across at a quicker rate, and 45-fold higher number than 75Q cells (d9 22Q = 503, 75Q = 11). These results suggested that further investigation was warranted into CPN axonal dynamics in baseline conditions.

This was further supported by the axonal projection efficiency observed between treatment groups of both 22Q and 75Q cultures (measure 3). In the 75Q cohort, NGF was able to dose-dependently increase the total number of projected axons, and thus projection efficiency above that of control (200 ng/mL = 27.1% vs control = 10.6%) (Figure 4.11G & H). However, when assessing 22Q cultures, although NGF did increase projection above other treatment groups, it
remained ineffective in comparison to control (200 ng/mL = 267 axons vs control = 503 axons) (Figure 4. 11L & M).

Finally, the axonal network formed post-projection was assessed and ranked according to the scoring system described in 2.9.2.1 (measure 4). For 75Q cultures, only the 200 ng/mL NGF treatment group was able to form simple axonal networks in which minimal interaction could be seen. Treatment with control media did not have any stimulatory effect, and treatment with either 22Q or 75Q MSN conditioned media appeared to hinder the formation of axonal networks (Figure 4. 11I). In contrast, 22Q cultures appeared much more able to form axonal networks in almost all treatment groups (Figure 4. 11N). Both 200 ng/mL NGF and control media treatment cultures produced complex axonal networks with multiple interaction sites. Interestingly, the data demonstrate that despite low axonal projection frequency, in the 75Q MSN conditioned media treatment group, a simple axonal network was able to form, greater than the complexity observed in 22Q MSN media treated cultures. This result was particularly interesting when compared to Figure 4. 11I, as it suggests that CPNs of different genotypes may behave differently when projecting towards either non-HD or HD MSNs as a result of, potentially, the trophic factors or cellular stress signals being released.
Figure 4. 11. Axonal projection of PSC-derived CPN-containing cultures after stimulation. One HD clone [75Q (3)] was seeded into the somal compartment of MFC and allowed to attach for 24 h. A full media change of the axonal compartment was completed with media containing either 50 - 200 ng/mL GDNF, 50 - 200 ng/mL NGF, 22Q MSN/ 75Q MSN media.
conditioned media or N2B27 (control) media. Somal compartments remained in N2B27 media throughout. Half media changes were completed every 48 h. Axon dynamics were assessed over a period of 10 days. Graphs represent the 4 measures assessed: Channel occupancy with (A) GDNF, (B) NGF and (C) MSN conditioned media; Axons crossed in (D) GDNF, (E) NGF and (F) MSN conditioned media conditions. The total number of projected axons at the termination of the experiment is also shown as both absolute numbers (G), and percentage axon crossing efficiency (H). The complexity of the axonal networks generated at the termination of the experiment and was scored from 0-3 (I). The same experimental set-up was performed using a non-HD clone [22Q (1)] with just NGF or MSN conditioned media as GNDF showed no effect previously (J - M). The projection of 22Q and 75Q axons in baseline conditions were re-plotted against each other to show the overall axon crossing efficiencies seen with these experiments (O). The colour key pertaining to each treatment group is shown. Data are presented as raw values; n=1 per treatment condition except MSN conditioned media and controls where data points are the mean (n=2) ± S.D.
4.5.5.3 Axon crossing ability of CPNs within MFCs

Following the results of both un-stimulated, and stimulated pilot experiment sets, a full investigation was designed to assess the axonal dynamics of the HD family series, by assessing their axonal projection over a period of seven days. As preliminary results suggested a mHTT-dependent phenotype may be present, experiments were conducted in PSC-derived 22Q, 58Q and 75Q CPN-containing cultures, so it could be identified if the phenotype was Q-length dependent. Furthermore, as the number of MFCs per clone was increased to increase the number of technical repeats, the number of measures assessed was reduced to focus on axonal projection frequency, latency and network formation.

As can be seen in Figure 4. 12A, there is a polyQ-repeat length dependent deficiency in the ability of cells to project through MFCs. At day five, 75Q cells were projecting on average 184 fewer axons than 22Q cells (p = 0.029). By day six, significant differences were observed between all groups [22Q vs 58Q p = 0.04; 22Q vs 75Q p = 0.0052; 58Q vs 75Q p = 0.037], which was further supported by day seven whereby both 22Q and 58Q cells had projected significantly more axons than 75Q cultures [22Q vs 75Q p = 0.455; 58Q vs 75Q p = 0.0014].

Secondary analysis consisted of imaging the axonal networks that were formed post-axonal projection. As can be seen qualitatively in Figure 4. 12B, 22Q cells produced highly dense and complex axonal networks. Axonal interactions were evident and on high magnification, it was possible to observe axonal swellings around interaction points. In contrast, the complexity of axonal networks produced by cells carrying the HD mutation appeared to be reduced in a CAG-length dependent manner (Figure 4. 12C & D). Efforts were made to identify the level of electrophysiological activity in these cultures and show that these were functionally active neurons, using the calcium binding dye Fluo4. However, despite extensive optimisation efforts, the difference in Z plane between micro-channels and somal/axonal chambers prevented clear time-lapse imaging of the cultures as a whole, therefore these data could not be used to assess connectivity.
Figure 4. Axonal projection is reduced in HD CPN-containing cultures in a CAG-length dependent manner. Neurons were cultured for a period of 7 days in MFCs and axonal projection was quantified every 24 h (A). Data are presented as mean ± SEM; (N = 1, n = 2, n = 4)* p < 0.05; ** p < 0.01 [* = 22Q vs 75Q; # = 22Q vs 58Q; $ = 58Q vs 75Q]. Representative images of axonal networks formed post-projection by 22Q CPNs (B), 58Q CPNs (C) and 75Q CPNs (D).

4.5.6 BDNF release and storage

In HD animal models, almost every aspect of BDNF processing is altered. Fewer studies however, have aimed to replicate this in a human model, therefore it is unknown as to whether the same phenotypes in terms of reduced BDNF production, deficient BDNF anterograde trafficking and subsequent release, as well as post-synaptic uptake and retrograde movement, are also replicated in a human context. This project therefore aimed to identify whether any HD phenotypes emerged with regards to BDNF in PSC-derived CPN-containing cultures and if so, whether in a HTT polyQ-length dependent manner.

4.5.6.1 BDNF release

Initial investigations focused on assessing the concentration of BDNF protein released by the CPN cultures into their supernatants. In the CNS, BDNF is released in an activity dependent manner from axon terminals, usually in combination with glutamate (Bathina and Das, 2015; Ferrer et al., 2000). Therefore, this project assessed BDNF release in both un-stimulated (baseline) and stimulated conditions. Two methods of stimulation were chosen: potassium chloride (KCL) is the classic stimulant used for BDNF release studies (Hartmann et al., 2001; Kuczewski et al., 2009; Zuccato and Cattaneo, 2007) as it depolarises cellular membranes and encourages the release of all BDNF from internal stores (Campbell, 2004). On the other hand, glutamate was used to assess excitation-induced release. The concentrations of each stimulant were relatively high for physiological studies, but were chosen on the basis of previously
published studies and to ensure the concentrations of measured protein would be within the detection limit of the ELISA used for assay.

The results from the BDNF release studies were somewhat challenging to interpret (Figure 4.13). Although there did appear to be a HD-associated phenotype, albeit not in an HTT polyQ-length dependent manner, the overall variation between experiments, particularly in control cultures, precluded a significant difference between the groups using two-way ANOVA. Furthermore, as the concentrations released were close to the lower detection limit of the assay, even with stimulation, with some recordings not reaching this lower limit, there was not enough confidence to draw firm conclusions with regards to BDNF release. Overall there was no significant interaction between HTT polyQ-length and treatment ($p = 0.648$). Furthermore, neither polyQ-length nor treatment reached significance independently ($p = 0.132$, $p = 0.504$ respectively). Similarly, post-hoc analysis did not produce significant differences between treatments or CAG-lengths ($p > 0.267 - 0.99$).

4.5.6.2 BDNF storage

BDNF release relies on both its production and subsequent processing, as well as functional trafficking, whereby BDNF is trafficked along microtubules in DCVs. Defects in these phenomena had previously been reported. To determine if a reduced intracellular pool existed in HD clones, the total levels of intracellular (stored) BDNF were assessed by ELISA. The corresponding cell pellets used to generate data in Figure 4.13A were lysed in RIPA buffer and subject to ELISA quantification. As shown in Figure 4.13B, HD cultures appear to have reduced levels of intracellular BDNF protein compared to control cultures, which are albeit more variable. Overall there was a significant effect of HTT polyQ-length ($p = 0.0326$), however this significance was lost during post-hoc testing; a trend to significance was observed in KCL treated cultures with a mean difference in protein concentration of 47.49 pg/μg between 22Q and 75Q cultures ($p = 0.1$). Whilst these data suggest the HD mutation may be impacting the production and storage of BDNF, there was still the possibility that DCV trafficking may be deficient in these cultures also. Unfortunately, it was not possible to obtain the materials required to transfect exogenous BDNF, or tag endogenous BDNF (eGFP/quantum dot), therefore BDNF trafficking studies were not carried out.
Figure 4. 13. Release and storage of BDNF in stimulated and un-stimulated conditions from day 50 CPN-containing cultures. A) The levels of BDNF secreted into the supernatant without stimulation or after addition of 50 μM KCL or 200 μM glutamate was assessed using a BDNF ELISA assay (BDNF released is normalised to total cell protein (pg/ug) from each sample). B) To quantify the precise levels of intracellular BDNF, the corresponding cell pellets from (A) were lysed and intracellular protein also subjected to BDNF ELISA. Protein concentrations were normalised to total protein concentration as determined by BCA assay, using the same cell lysate. Data are presented as mean ± SD; (N = 1, n = 1-3, n = 8).

4.6 Discussion

To assess the possibility of mHTT-dependent phenotypes in developing CPNs, a multistage process was undertaken. Initially, a full characterisation of PSC-derived CPN-containing cultures was necessary to ensure the cells were representative of cortical layer V neurons found in the human CNS, prior to any assessment of phenotype or function. To that end, novel adaptations were made to the Shi et al protocol so that layer V CPNs could be reliably generated and assessed in multiple formats. Once characterisation of culture composition using immunofluorescence-mediated microscopy was completed as far as possible, several investigations were initiated into the functionality and phenotype of the cultures. These focused
on cell morphology, axonal outgrowth dynamics, and BDNF production and release; several mHTT-dependent phenotypes were uncovered.

4.6.1 Layer V CPNs can be produced from HD family series, and IsoHD series PSCs

The Shi et al protocol has been widely used in a variety of studies, producing cortical neurons for assessment of phenotypes for a multitude of diseases including HD. During the course of this project however, it was discovered that the reliability of differentiation success was not sufficient to enable robust investigation into HD phenotypes. As this was one of the primary aims of the project, adaptations were designed and carried out to increase the reliability of differentiation success and thus increase the material generated for experimentation.

The adaptations acted to reduce the levels of cell death experienced during the differentiation procedure, whilst also improving the plating distribution for terminal experimentation. It was observed that the adaptations did not appear to have any effect on culture composition, in terms of cortical layer production, as a significant proportion of layer V CPNs were easily identified in each differentiated culture. Furthermore, there did not appear to be any difference introduced by genotype in response to the adapted protocol i.e. both control and HD cultures responded equally.

The reliable generation of layer V CPNs enabled the assessment of culture composition between genotypes. It has previously been reported by several groups that the HD mutation hinders the differentiation and maturation of striatal cells (Conforti et al., 2018; The HD iPSC Consortium, 2012). However, this phenomenon has been relatively under-investigated in the literature with regard to CPNs, indeed the only reports found to date were published in 2018 (Conforti et al., 2018; Mehta et al., 2018). One study compared CPNs derived from juvenile HD lines expressing 180, 109 and 77 HTT CAG repeats with age matched controls. The results found in this thesis compliment those found by Mehta et al, in that no difference was observed between genotypes in the ability to produce layer V CPNs (Mehta et al., 2018). Moreover, the authors did not state any difference in the percentages of proliferating cells and NPCs in cultures when assessed over a variety of time-points. This study supports the first statement, in that no difference was observed in the percentage of proliferating cells at d50 as identified by ki-67+ staining, however a significantly increased percentage of nestin+ cells was observed in these HD cultures, suggesting that there could be variability between PSC lines. The authors also stated that the emergence of GFAP positive cells was similar between genotypes, however no evidence of GFAP reactivity was observed in the cultures used in this thesis. The reason behind this could be due to the time over which cultures were maintained - GFAP reactivity is not observed in cultures differentiated using the Shi et al., protocol until d100+. As the majority of cultures assessed for culture composition were terminated at d60, with the exception of d90 cultures assessed for upper cortical layer markers, it is not surprising that GFAP+ cells were not identified. Furthermore, without a positive control, it is not clear whether the antibody was functional or specific.
Whilst the authors of the 2018 study did not identify any differences in the ability of PSCs to differentiate into layers of the cortex, a phenotype was found in terms of neuronal maturation. Transcriptomic data revealed HD CPNs had lower expression of genes necessary for action potential generation, including sodium ion channels, than control neurons, thus suggesting that at the same time in culture, HD CPNs are less mature (Mehta et al., 2018). This project unfortunately could not assess the maturation rate of CPN-containing cultures despite multiple attempts, as the optimal conditions required for repeated MEA recordings could not be established and only incomplete data sets were produced.

4.6.2 The emergence of multiple HD-associated phenotypes can be observed in culture

Once fully characterised, investigations into the emergence of HD-associated phenotypes in culture could begin. One of the first parameters assessed was that of cell viability, as it has been widely reported in the HD brain the cortex is selectively vulnerable to mHTT toxicity, specifically layer V CPNs (Han et al., 2010). Using multiple methods, this project identified that PSC-derived CPN-containing cultures expressing mHTT had reduced viability when compared to control counterparts. These measurements were carried out in baseline conditions with no stimulation or stress and demonstrate that cortical vulnerability in HD is replicated in an in vitro format. Despite it being well established in the HD field that distinct layers of the cortex are also selectively vulnerable in HD (Han et al., 2010), a finding that is robustly reproducible in animal models (Ferrante, 2009) and supported by human data from post-mortem analysis (Estrada-Sánchez and Rebec, 2013), it was yet to be established whether PSC-derived CPNs show decreased viability in culture. Although the reduction in viability was small, these are important findings as it suggests that even in the earliest stages of disease, the HD mutation may be compromising CPN viability in the CNS and thus potentially impacting cellular function over a longer time course i.e. if the basal death rate is just 1% more than in controls, over time that may have a significant effect in terms of both structure and function.

As described previously (section 3.6.2.2), it has been reported that multiple cell types expressing mHTT have altered adhesion (Reis et al., 2011). Here, it was identified that PSCs and PSC-derived cortical NPCs showed reduced adhesion in a mHTT dependent manner. Interestingly, the difference in cellular adhesion between genotypes was lost once cells reached maturity at d50. These data were reflective of what was observed in routine tissue culture: the time taken for cells to detach during Accutase passage was equal between genotypes (~5 min) when performing the late-stage split. Furthermore, unlike MSNs, there did not appear to be any difference in the levels of detachment from the substrate during ageing - HD cultures were routinely maintained for 100+ days alongside control cultures. To identify the cause of the developmental stage-dependence of the adhesion phenotype, it would be interesting to assess the expression levels and localisation of integrins and N-cadherins in cell samples. By assessing levels of N-cadherin, it could be identified if these data truly corroborate those findings in Reis et al (2011). Moreover, as N-cadherin is intrinsically important for neurite outgrowth and actin cytoskeletal dynamics, in both NPCs and neurons (Liu et al., 2018;
Miyamoto et al., 2015), altered expression levels could also explain several other HD phenotypes as discussed below.

The morphology of neurons is extremely important for their correct functioning; axons need to be dynamic and as such, their microtubules and actin filaments need to polymerise quickly to reach their intended targets, as well as remodel themselves efficiently during learning and memory. As the cortical layer V neurons form some of the longest projections within the brain (Haber, 2016; Reiner et al., 2010), it was important to identify whether the expression of mHTT altered the morphological properties of these neurons. By using a longitudinal imaging platform, it was possible to not only visualise the cultures in controlled conditions over time, and therefore complete comparisons at each time point, but also to assess any differences in the rates of change over time. The results from these experiments proved intriguing, more so as they were somewhat in line with previously published findings. Although no significant difference was observed in the overall percentage change of neurite length between genotypes, it cannot be overlooked that at every time-point assessed, HD neurites were on average 10 mm shorter in length than control counterparts, so much so that at the final time-point assessed, the average neurite length of 75Q cultures reached the same value as 22Q cultures at the start of recording. This could indicate that the HD mutation renders the neurons less able to extend neurites in a global manner. These results compliment similar studies, in which Neuroleucida tracing software was used to measure the neurite length of iPSC-derived CPNs differentiated with the un-modified Shi et al protocol (Mehta et al., 2018). The authors identified that at d130 in culture, there was an HTT CAG-length dependent decrease in neurite length of MAP2+ CPNs. Therefore, it would be interesting to repeat the experiments in this thesis at multiple time points, to identify if the deficiency in neurite length progresses over time.

The data presented here did not entirely support the study led by Mehta et al., (2018), however, in that the authors categorised the neurons within each culture into either an 'un-branched', 'branched' or 'multipolar' state, and found that there was no difference in the proportions of each category between genotypes. Although this project did not categorise the overall morphology of cells, it was observed that the rate of change in number of branch points trended towards significance, with HD cultures producing fewer branched neurites than control counterparts. A possible explanation for this difference in result could be due to the age at which the measurements were recorded. These data were derived from d50 cultures, which are still relatively immature and dynamic in their properties, whereas the data produced by Mehta et al was collected from neurons aged to 130 days. Furthermore, as the analysis methods differed, it is difficult to compare the results of the two studies.

One aspect of CPN morphology that has been well studied in the past is that of neural polarity, and whether the HD mutation may impact the ability of CPNs to migrate throughout the layers of the cortex to their intended terminal location. Indeed, several studies have identified that HTT has a role in mitotic spindle orientation during development (Godin et al., 2010b; Molina-Calavita et al., 2014), plus a study conducted by Barnat et al., in 2017 discovered that the multipolar-bipolar switch that is required for cortical migration was deficient in HD and resulted
in abnormal cortical layering (Barnat et al., 2017). There is a relative lack of information however, on the axonal dynamics and actin cytoskeleton functionality in CPNs in HD. Due to the abnormalities in cellular adhesion, as well as the altered morphological state of HD CPNs, a series of experiments were designed in an attempt to assess the axonal and actin dynamics of CPNs in a quantitative manner.

Using MFCs, it was possible to visualise the projection of axons between distinct chambers, and thus assess axonal projection frequency and latency. The results proved extremely interesting and are a novel finding, as it was observed that axonal projection was deficient in HD in an HTT polyQ-length dependent manner. Indeed, the majority of the data published on the CPN CS tract is derived from imaging data, which is of course sampled from human patients, or animal models once the pathway is fully formed (McColgan et al., 2015). Once projected, it was also observed that the axonal networks formed, differed in their density and interactivity between genotypes; axonal network morphology also appeared to differ in a polyQ-length dependent manner, with increasing polyQ-length producing less dense and less interactive networks.

These data raise an interesting question that is currently highly debated in the field: does HD have a neurodevelopmental element?

It could be argued that if there is deficient CPN axonal projection in the HD brain, pathways such as the CS pathway could either be delayed in their formation, or may never form correctly. Against this hypothesis, is the fact that HD gene carriers typically remain symptom free until the fourth decade of life (on average) and seemingly develop near-normal cortices. The effects of any developmental differences therefore must be slight, to the extent that they are easily compensated for. However, the fact that both brain regions connected by this pathway i.e. the cortex and the striatum, unequivocally develop pathology first, could indicate that if indeed dysfunctional projection is compensated for, this is a limited process; whether the limitation is HTT polyQ-length dependent, or simply time dependent, remains to be addressed. Regardless of this complex argument, the timeline of CS pathway formation has important implications for the most vulnerable cell type in the disease, MSNs. It is well established that MSNs rely on the neurotrophic support provided by other brain areas, with BDNF being one of the key neurotrophins required for their correct differentiation, survival and functionality (Ferrer et al., 2000). Animal studies have identified that during neurodevelopment, CPNs do not begin producing BDNF until postnatal (P)4 (Baquet et al., 2004), whereas the substantia nigra begins expression as early as embryonic (E)13.5. However, as nigrostriatal connections are evident at E16.5, the time point at which BDNF protein is also observed in the striatum, it is suggested that in the earliest stages of neurodevelopment the striatum receives the majority of its neurotrophic support from other brain areas, as the CS pathway has not fully formed (Baydyuk and Xu, 2014). However, once formed, the CS pathway 'takes over' and produces the majority of trophic factors required for MSN functionality. If the CS pathway does not form correctly, or MSNs endure a period during later neurodevelopment where these support factors are not being delivered, it could be suggested that this may partially explain the selective vulnerability of MSNs in HD. Furthermore, if the successfully projected axons are less interactive, it is plausible
that fewer MSNs would receive stimulation, and thus support, from a single CPN therefore reducing the complexity of the CS network. However, this is just one of several plausible explanations. Extensive further study would be required to confirm these hypotheses, and as such is beyond the scope of this thesis.

Another aspect of axonal dynamics that was investigated here was the pathfinding ability of CPNs. By using a variety of stimulants, including NGF, GDNF and the conditioned media of MSNs, it was possible to identify if there was any discrepancy in the ability of cells to successfully project a leading neurite into micro-channel openings, through the micro-channel and into the axonal chamber. The choice to use NGF and GDNF as stimulants for axon crossing was based on a literature search of compounds commonly used to stimulate neuronal projection, both GDNF and NGF were identified as key chemoattractants for CPNs (Drescher, 2011; Markus et al., 2002). By applying the treatment to the axonal chamber only and maintaining fluidic isolation within the device during the experiment, it was possible to assess only the retrograde signalling and consequent projection, rather than somal feed-forward signalling. When analysing the results, several interesting observations were made. At the first time-point, 22Q cells appeared to project triple the number of axons into micro-channels than 75Qs, regardless of any treatment. As the seeding density was controlled for, this suggests that the rate of initial projection is much greater in non-mHTT expressing cells. Interestingly however, when the rate of continued projection is followed over time, 75Q cells appear to increase the number of channels occupied in an exponential manner, whereas 22Q projection follows a more linear trajectory, again regardless of treatment. This could suggest that pathfinding ability in cells expressing mHTT is simply delayed rather than deficient. However as this rate plateaus around d5 of treatment, it is likely that extending the treatment would not result in 75Qs ‘catching up’ with 22Q neurons. This was confirmed by the number of channels occupied at the termination of each experiment, as in some treatment groups, 22Q cells still occupied more than triple the number of micro-channels than 75Q neurons.

In order to identify if this phenotype was indeed caused by a reduction in the dynamics of actin polymerisation, or was due to altered growth cone kinetics, future work could focus on live-imaging experiments in which actin was fluorescently tagged. This would enable the visualisation of actin polymerisation and depolymerisation in real-time, and thus reveal if axonal projection and retraction was indeed slower in cells expressing mHTT. Furthermore, it would also be interesting to conduct non-restricted migration assays, i.e. not in MFCs, to identify if somal migration also appeared to differ between genotypes, as this could further support the hypothesis that the formation of the CS pathway may be delayed in HD. CPNs must migrate to their terminal location prior to beginning axonal projection (Sohur et al., 2014), therefore if this initial process is delayed, axonal projection and synaptogenesis with targets such as MSNs could be impacted. As the growth cone is integral to both of these processes (Murphy et al., 2000; Tousley and Kegel-Gleason, 2016), investigations into the responsiveness of growth cone dynamics to trophic factors would be of extreme interest. Indeed, this is a relatively under investigated area of HD pathophysiology, and as such future experiments would produce novel findings in the field.
Processes including axonal transport and microtubule trafficking have been found to be heavily affected in HD (Akbergenova and Littleton, 2017; Bates et al., 2015; Ehrlich, 2012; Ferrante, 2009; Labbadia and Morimoto, 2013). This is further compounded by a role for WT HTT in axonal transport, whereby the mutant form causes a loss-of-function phenotype (Saudou and Humbert, 2016). As aforementioned, BDNF is a key neurotrophic factor required for MSN survival and differentiation. Contained in DCVs, BDNF is trafficked along CPN microtubules prior to its release at axon terminals in an activity-dependent manner. Data presented here have demonstrated that axonal projection is deficient in HD, therefore investigations were made as to whether this had downstream effects on BDNF secretion.

Although the results did not reach significance, and therefore must be interpreted with caution, there did appear to be a difference in the concentrations of BDNF released into cell culture media depending on genotype. Both lines expressing mHTT released lower concentrations of BDNF than control counterparts, irrespective of stimulation. This is a common finding in the HD field (Estrada-Sánchez and Rebec, 2013; Huang et al., 2016; Zuccato and Cattaneo, 2007), indeed several groups have teased out the cause of this and found multifactorial mechanisms of action. The release of BDNF relies on at least three factors: the production and storage of BDNF, BDNF trafficking, and vesicle fusion and subsequent release. This project found that there was a significant reduction of stored or intracellular BDNF when assessed by two-way ANOVA, however significance was lost during post-hoc testing. Unfortunately due to the inability to obtain expression constructs, BDNF trafficking could not be assessed and further comment on the BDNF trafficking deficit described in other studies cannot be made. Furthermore, despite precise experimental procedure and rigorous controls, the variability observed between clones and experiments limits the significance that can be drawn from the data presented here. The chosen method, a commercially available BDNF ELISA, had the highest sensitivity of commercial assays available. However, to better quantify the levels of BDNF production both in terms of protein and mRNA expression, other techniques such as western blotting and quantitative PCR could be employed. As MFCs in their entirety are amenable to imaging, it would be valuable also to conduct live-imaging of tagged BDNF within micro-channels so that the velocity of vesicle trafficking could be assessed.

There have been many studies into the impact of BDNF over-expression, deficiency, exogenous administration and depletion in HD, as it has high potential as a therapeutic target not only for this disease but multiple other neurological diseases. Experiments have been published that investigate the exact processes that contribute to a reduction in BDNF trafficking, such as vesicle number (Virlogeux et al., 2018) and trafficking velocity (Baydyuk and Xu, 2014) and also suggest a possible explanation for reduced BDNF trafficking in mHTT-expressing neurons (Drouet et al., 2014). Also, several groups have studied the expression levels of both the BDNF mRNA transcript as well as the quantity of protein produced (Gauthier et al., 2004; Apostol et al., 2008; Baydyuk and Xu, 2014; Ferrer et al., 2000). These studies will be discussed in further detail in Chapter 6, as they are highly relevant to the future directions of this project.
4.7 Limitations
Whilst every effort was made to ensure as pure a neuronal population as possible, it cannot be overlooked that full identification of culture composition was not achieved for PSCs subjected to the cortical differentiation protocol. A variety of different neuronal types were identified within cultures, including a proportion of cells that expressed the markers identifying layers II – IV. Similarly to the MSN differentiation protocol however, the identity of some cells within cortically differentiated cultures remains unknown and has similar connotations to the previous chapter; it cannot be guaranteed that the phenotype observed is due to the true CPNs or NPCs within the culture, or indeed an artefact or result driven by the ambiguous cells within cultures. This has been identified as a limitation of this thesis.

4.8 Summary
An optimised protocol for the generation of layer V CPNs was produced, that could reliably produce cultures in which a percentage of CTIP2+ neurons could be identified. Full characterisation of these cultures demonstrated characteristics akin to layer V CPNs found in the brain, thus it was assumed that experimental outcomes could be representative of processes occurring in the human CNS. A variety of HD-associated phenotypes were observed in culture, suggesting that the HD mutation has an impact of cellular functioning in pre-pathology. It was observed that cell viability was reduced with the expression of mHTT and several functional aspects of CPN development were also affected. The morphology and adhesion ability of CPN-containing cultures was found to differ between genotypes, plus a novel experiment revealed axonal projection is deficient in HD cultures in a CAG length dependent manner. Finally, preliminary evidence suggests that BDNF levels are affected in PSC-derived CPN-containing cultures; the concentration of BDNF released in either baseline or stimulated conditions was lower than control cultures, which could be partially explained by the significant reduction of BDNF protein found to be stored intracellularly. In summary, these data provide partial confirmation of results reported in previous literature, as well as contributing novel findings with regard to CPN axonal dynamics.
Chapter 5 - Generation of a co-culture platform to model the corticostriatal pathway in vitro

5.1 Background

Within the HD field it is widely acknowledged that the CS pathway is of particular interest, as the cell types comprising it, namely the layer V CPNs and striatal MSNs, are the ones which principally degenerate in the disease (Bates et al., 2015). Functionally, the CS pathway is key to multiple aspects of human behaviour. By regulating the activity of the thalamus, and subsequent projection to the motor cortex, the CS pathway facilitates coordinated movement and fine motor skills (Haber, 2016). Furthermore, as the cerebral cortex predominantly projects to the striatum, including both from frontal and parietal lobes, there is a longstanding hypothesis that the CS pathway is also involved in cognition (Penrod et al., 2015).

It comes as no surprise therefore that both of these faculties are disturbed in HD patients (Bates et al., 2015). The classic choreic movement is the cornerstone of an HD diagnosis and is caused in part by the neurodegeneration of the striatum and subsequent loss of forward-processing to the motor cortex. A breakdown in CS connectivity is believed to directly contribute to this (Bunner and Rebec, 2016; Burgold et al., 2019; Cattaneo, 2007). With regards to the cognitive symptoms of HD, many groups have suggested that altered CS connectivity as a result of mHTT toxicity contributes to deficits in executive function, working memory and visuo-spatial dysfunction, sometimes very early in the disease. Indeed, evidence from the PREDICT-HD clinical trial shows cognitive changes can precede the onset of motor symptoms by a decade (Stout et al., 2011), which indicates that CS pathway dysfunction is in fact an extremely early event in HD pathogenesis. Much focus has been placed on understanding exactly what occurs in HD patient brains that leads to the loss of CS pathway connectivity, because the CS pathway could be a key target for early therapeutic intervention (Huang et al., 2016; Wild and Tabrizi, 2014).

Human CNS imaging data has greatly contributed to our understanding of CS connectivity breakdown during disease progression. For example, it was found that prodromal HD patients (~10.4 years from onset) could be distinguished from control subjects based on their CS connectivity as assessed by BOLD fMRI (Unschuld et al., 2012). Moreover, animal models of HD have demonstrated disrupted CS connectivity, which worsens over time with disease progression when assessed by electrophysiological studies (Cepeda et al., 2010, 2007; Estrada-Sánchez and Rebec, 2012). Joshi et al identified that alterations in CS connectivity at the cellular level, in terms of synaptic connectivity and glutamate release, were altered by one month of age in YAC128 mice, which precedes the age at which an HD behavioural phenotype manifests in this model (Joshi et al., 2009). Similarly, in the Q140 mouse model, Deng et al found a reduction in the absolute number of CS synaptic terminals prior to the onset of striatal pathology, suggesting that dysfunction of the CS pathway may commence at the synaptic level (Deng et al., 2013).
Some groups have attempted to model the CS pathway in vitro using primary neurons harvested from HD animal models. The use of primary neurons for these experiments is advantageous as they are extremely well characterised (Milnerwood et al., 2012; Shehadeh et al., 2006), and exhibit HD-associated phenotypes such as mHTT aggregates (sections 3.1 and 4.1). The principle way in which CS pathways have been reconstituted is by co-culturing ex-vivo layer V CPNs and MSNs. A variety of different co-culture methods have been generated which typically fall into three categories: mixed culture, shared media, or limited physical interaction platforms. Mixed culture models either combine cell suspensions of dissociated CPNs and MSNs before plating on a communal coverslip, or plate one cell type directly on top of the other sequentially, again in a communal culture vessel (Milnerwood et al., 2012; Penrod et al., 2015; Buren et al., 2016). In 2012, Milnerwood et al., developed mixed population co-cultures derived from WT and YAC128 mice. Focusing primarily on electrophysiology and glutamatergic signalling, the authors identified that YAC128 cultures exhibited increased surface expression of the GluN2B NMDAR subunit, whilst GluN2A expression remained unchanged between conditions. This led to an increased extrasynaptic NMDAR current within HD cultures, which had downstream effects on both NMDA-mediated apoptosis and pCREB shut off, which were increased by 25% and 35% in HD cultures respectively. Using optogenetic techniques, Artamonov et al., selectively stimulated WT and YAC128 CPNs co-cultured with MSNs and identified a disrupted synaptic transmission in YAC128 cultures; the latency for excitation was much greater in YAC128 CPNs compared to WT, which had downstream effects on MSN excitability. Interestingly, when assessing CPN and MSN cultures independently, the authors noted that YAC128 CPNs exhibited a hyperexcitable phenotype which persisted for three weeks in culture, before decreasing to below WT levels. Similarly, when assessing dendritic spine morphology and density in MSNs, the numbers of mature 'mushroom' spines appeared similar between HD and WT cultures until ~d19, at which point mushroom spines began disappearing, replaced with the immature 'thin' spines. The fact that these phenomena occur in tandem led the authors to the hypothesis that a cyclical degeneration is occurring in co-cultures - a decrease in cortical activity reduces the probability of mushroom spine maintenance, causing their degeneration, this ultimately leads to reduced cortical transmission and causes a further reduction in cortical activity (Artamonov et al., 2013).

Shared media only paradigms prevent physical interaction of the two cell types, either by co-culturing in a device with separate chambers and a porous membrane, or by seeding individual neural populations on separate coverslips which are then placed within a larger culture vessel (Penrod et al., 2015). Both of these methods enable the free diffusion of solutes and trophic factors into the shared medium, without physical contact ever occurring between the two neuronal populations. Finally, limited interaction models, which have been designed and utilised more recently, allow for a directionality of interaction to be introduced into co-cultures. Two methods have been described. Garcia-Munoz et al designed a system in 2015 that was not used in a HD context. The authors cultured CPNs and MSNs harvested from E14-17 mice in separate compartments of a culture vessel - the separation was introduced by using a cell culture insert that could be removed when desired (Ibidis, Thistle Scientific). Once the neuronal
populations were established individually, the culture insert was removed and authors observed a rapid unidirectional projection of CPN axonal processes towards MSNs which formed functional synapses, confirmed by MEA and patch clamp recordings (Garcia-Munoz et al., 2015). The second method is considered more robust, and utilises MFCs.

MFCs, as discussed in section 1.9.3.2, are devices that incorporate a physical separation into a culture system, and can also introduce fluidic isolation when a volume differential is in place (Park et al., 2006). By treating the somal compartment and axonal compartment as cortical and striatal regions respectively, one can attempt to replicate the physical separation that occurs in the brain and recapitulate the neurodevelopmental processes involved in first establishing CS connectivity. Whilst cell bodies are separated by the chamber walls, a series of micro-channels connect the chambers, which allow for axonal projection and subsequent connectivity. Co-cultures using this experimental design have proven fruitful with regards to identifying HD phenotypes in the CS pathway, as well as confirming results previously identified in intact systems from animal models (Kaufman et al., 2012; Milnerwood et al., 2012; Virlogeux et al., 2018).

Zhao et al., assessed synapse formation and aspects of BDNF functionality in co-cultures derived from primary BACHD neurons seeded in MFCs. The authors identified a reduction of synapses in co-cultures that contained BACHD CPNs, irrespective of MSN genotype, suggesting that mHTT expression in the presynaptic neuron was sufficient to initiate disease pathology. The second main phenotype was that BDNF trafficking in both the anterograde and retrograde direction was impaired in BACHD co-cultures. The authors went on to identify that treatment with a subunit of TriC (a cytosolic chaperone) restored retrograde transport to WT levels (Zhao et al., 2016).

Finally, Virlogeux et al., developed a tri-chamber MFC comprising two somal compartments in which primary WT or HdhQ140 CPNs and MSNs were seeded, and a third synaptic compartment which was located 450 μm and 75 μm from CPN and MSN somal compartments respectively. By measuring synaptic phenotypes only from this chamber, the authors ensured that only CS synapses would be recorded, as the distances between chambers ensured only cortical axons, and MSNs dendrites could interact. The authors reported that both anterograde and retrograde transport of BDNF was compromised in HD co-cultures. By transfecting cultures with fluorescent indicators iGluSnFR and GCaMP6f, the authors identified a disrupted glutamatergic transmission between neuronal populations, which was supported by a reduced number of synapses observed in HD co-cultures. In the striatal somal chamber, an interesting result was found in the activity and synchronicity of neuronal firing, as HD cultures exhibited hypersynchronised burst firing, but were otherwise largely inactive in comparison to WT cultures. Finally the authors constructed chimeric cultures in a similar format to above, and identified that the phenotypes described were dependent on the genotype of the presynaptic compartment i.e. the CPNs. In cultures where CPNs expressed mHTT, the HD-associated phenotype was observed, irrespective of the genotype of MSNs, thus supporting the finding by Zhao et al (Virlogeux et al., 2018).
The use of human neurons within these devices, especially of PSC origin had not yet been investigated however. This project was therefore the first known report in which a model of the human CS pathway was constructed using PSC-derived CPNs and MSNs, enabling investigation of human HD-associated phenotypes.

5.2 Aims
1. To establish the viability of PSC-derived MSNs and CPNs when co-cultured in a mixed population.
2. To assess the indirect effects of mHTT on culture composition and viability, by applying conditioned media from PSC-derived CPNs on MSNs.
3. To co-culture PSC-derived CPNs and MSNs in MFCs to recapitulate the formation of the CS pathway in vitro.
4. To test expanded HTT polyQ-dependent effects in terms of synapse formation and viability of pure or chimeric CS co-cultures.

5.3 Methods
A summary of methods used in this chapter are outlined below. Full experimental protocols and parameters can be found in Chapter 2 - Materials and methods.

Pluripotent stem cells were cultured as described in section 2.2 prior to differentiation into MSNs and CPNs as described in sections 2.3 and 2.4.

A variety of co-culture paradigms were designed that included mixed population co-cultures, conditioned media paradigms as well as MFC-based co-cultures.

5.3.1 Mixed population co-cultures
Ibidi culture vessels are optically clear and allow for a larger diameter culture surface than coverslips. HD family series MSNs were seeded into Ibidi culture vessels at either 1 x 10^5 or 2 x 10^5 cells per vessel and left to attach for 24 h. As PSC-derived MSN- and CPN-containing cultures are similar in morphology, the MSN-containing cultures were then subject to lipofectamine-mediated GFP transfection as described in section 2.5.6 to enable easier identification. For half of the cultures, an additional 1 x 10^5 PSC-derived CPN-containing cultures were seeded directly onto the adhered MSNs. As MSNs required media supplementation with Activin A, GDNF and BDNF, it was decided that this would be the media in which co-cultures were maintained. Mono-cultures (MSNs only) and co-cultures (MSNs + CPNs) were maintained for five, or ten days, and observed daily using brightfield microscopy for signs of overt cell death. At day 5, brightfield images were acquired at 10x magnification. At day 10, brightfield images were again acquired prior to culture fixation and confocal microscopy as described in section 2.5. As only MSNs were transfected with GFP, a 488 Alexa Fluor-conjugated secondary antibody was targeted against DARPP-32; this marker is largely specific for MSNs therefore the same channel was used in order to leave the remaining channels open for further data collection.
5.3.2 Conditioned media paradigm
Experiments in which MSN-containing cultures were incubated with conditioned media collected from PSC-derived CPN-containing cultures were completed as described in section 2.10.1.

5.3.3 MFC-based CS co-cultures
To physically recapitulate the CS pathway, MFCs were used and co-culture experiments were run as described in sections 2.10.3 and 2.10.4.

Brightfield microscopy was used to monitor MFC-based co-cultures, and immunofluorescence was used as described in section 2.5 to visually assess the levels of cell death.

A pilot experiment was completed in which HD family PSC-derived CPN-containing cultures with HTT polyQ-lengths were co-cultured with control MSNs as described in section 2.10.4.1, prior to a full experiment as described in section 2.10.4.2.

To quantitatively assess cell viability, LDH experiments were completed as described in section 2.10.4.3.

Finally, assessing the formation of synapses was completed using the standard immunofluorescence protocol (2.5.2) that had been adapted for MFC culture vessels. Briefly, MFCs were tilted to 45° to allow draining of the culture media by gravity. Supernatant (if not used for LDH assays) was aspirated and 150 μL PBS added to both of the top wells of each MFC to wash remaining culture media from the chambers. After aspirating the PBS from bottom wells, 150 μL 10% formalin solution was applied to both of the top chambers and allowed to pass through the chambers with gravity. The cultures were fixed for 15 min prior to aspiration of formalin from the bottom wells. The devices were washed twice with 150 μL PBS by adding to the top wells, and aspirating from the bottom wells. Devices were then used immediately for immunofluorescence staining. To permeabilise the cells, 0.2% Triton-X 100 in PBS was added to the top wells and incubated at RT for 15 min. After aspiration from bottom wells, non-specific binding was blocked with 10% donkey (dk) or goat (gt) serum (dependent on secondary antibody host species), and 1% BSA in PBS for 1 h at RT. Primary antibodies were diluted appropriately (Table 4) and 150 μL was added to both the top wells and incubated for 2.5 h at RT. Primary antibody solution was removed from the bottom wells and a series of washes were completed in which 150 μL PBS was added to the top well and allowed to drain through the chambers due to gravity where it was the aspirated from bottom wells. This procedure was completed at least five times. Alexa Fluor-conjugated secondary antibodies corresponding to the correct species were diluted 1:1000 in PBS and added to top wells and incubated in the dark for 45 min. Secondary antibodies were washed in PBS as described above three times prior to a 5 min incubation of 1 μg/mL Hoechst in PBS also protected from light. Three final rounds of washing were completed prior to storage at 4°C, or immediate use. This process was completed at day 22 (3 weeks co-culture) and day 30 (4 weeks co-culture).

Airyscan (Zeiss) processing was applied to acquired images, and .czi files were converted to JPEG format. Processed images were imported into Fiji (is just) ImageJ software (version
the PunctaAnalyzer plug-in (Ippolito and Eroglu, 2010) was used as follows: JPEG images were opened and a selection was applied to the entire FOV. PunctaAnalyzer was launched, the co-culture name was set as condition, and red channel, green channel and subtract background check-boxes were ticked. Rolling ball radius was set to 50.0 pixels, and a threshold (range 65-255) was applied to the red channel image. Particles were analysed of sizes (pixel$^2$) 4-Infinity, circularity 0 - 1. The same parameters were then applied to the green channel, and co-localisation of ‘red’ and ‘green’ puncta within a specific distance from each other and if the parameters are met, a synapse is counted. Unfortunately the plug-in does not provide a masked image of the resulting synapses therefore user verification and quality control could not take place. Results were saved in Microsoft Excel. At least 3 FOV were selected per clonal co-culture.

Statistical analysis was completed as described in section 2.11.

5.4 Contributions

N/A

5.5 Results

5.5.1 Mixed neuronal co-cultures

Whilst this project was not investigating the CS pathway using a mixed culture design, it was necessary to identify if the two neuronal populations used here could survive in the same culture media, as the ratio of components and supplementation of N2B27 and MSN post-26 media, differed. As shown in Figure 5. 1A - H, no overt cell death phenotype was observed when cultures were maintained in MSN post-26 media, this was concluded as neuronal morphology was clearly evident, and although cellular debris was seen, the majority of the cells in cultures were phase-dark and adhered to the culture substrate. After five days culture, Figure 5. 1A and C depict MSN-containing mono-cultures, whilst B and D depict co-cultures with PSC-derived CPN-containing cultures. Co-cultures were then maintained for a further five days whereby brightfield images were once again acquired. When observing the impact of seeding density on culture morphology and phenotype, there was not an obvious difference after five days of co-culture; more densely seeded MSNs (Figure 5. 1C and D) had a similar cellular distribution and morphology to those with $1 \times 10^5$ MSNs and CPNs (Figure 5. 1A and B) and levels of phase-bright cells (dead cells) appeared similar between cultures. After ten days co-culture subtle differences were observed. Figure 5. 1E and F show sparser cultures which was not surprising due to the lower seeding density, however the number of projections and complexity of interactions appeared reduced when compared to Figure 5. 1G and H in which double the number of MSNs were seeded. The higher level of network complexity could suggest that more inter-neuronal connections were formed, however as functional activity was not investigated in these experiments, this cannot be confirmed.

As the two neuronal populations could not be easily distinguished due to similar morphology, the cultures were fixed after ten days co-culture and subject to immunofluorescence staining.
Prior to CPN-culture addition, MSN-containing cultures had been transfected with GFP (Lonza pmaxGFP) so that a proportion of MSNs would express GFP. Cultures were therefore incubated with antibodies targeting DARPP-32 (in the green channel, to cross-label with GFP) and CTIP2 as shown in Figure 5. 1I - J, and show neuronal cells co-expressing DARPP-32 and CTIP2 (with or without GFP) as MSNs, and cells expressing CTIP2 alone as layer V CPNs.

Taken together these results suggest that the maintenance of CPN-containing cultures in MSN post-26 media does not overtly affect their viability; they are able to survive in media with subtly different component ratios than suggested by Shi et al (2012). Also, these data suggest that the addition of CPNs to an MSN culture environment does not negatively affect their morphology or viability. It should be noted that as these were preliminary experiments, they were conducted with PSC-derived neurons that did not express pathogenic HTT polyQ-lengths, and results may differ if conducted with pathogenic lines.
Figure 5.1. Mixed population CS co-cultures. HD family PSC-derived MSN- and CPN-containing cultures (22Q) were co-cultured together for 5 (A-D) or 10 (E-H) days in MSN post-26 media. Cultures were seeded at 100k (A, B, E, F) or 200k (C, D, G, H) density. Images A, C, E and G show MSN-containing mono-cultures, and images B, D, F and H show co-cultures comprising and additional 100k or 200k d50 CPNs. MSNs were GFP transfected prior to plating, co-cultures were fixed after 10 days incubation and assessed for DARPP-32 (green, with GFP), CTIP2 (red), and Hoechst (blue), example images of which are shown in I and J. Scale bars represent 50 μm.
5.5.2 Conditioned media paradigm

To assess if any factors secreted by CPNs into the medium, such as neurotrophins, or any alteration to the properties of the medium that could have an impact on MSN functionality, viability and phenotype, a pre-conditioned media experiment was designed. PSC-derived MSN-containing cultures of varying HTT polyQ-length (22Q, 30Q and 75Q) were seeded into PE plates, and at d36 underwent a 90% media change into conditioned N2B27 media collected from 22Q, 45Q and 75Q CPNs aged to 50 days - 10% of supernatant was maintained on the cells to prevent detachment. Two control (supplemented MSN post-26, and N2B27) media were also tested. After 48 h co-incubation, MSN cultures were assessed for LDH release as well as HCl to assess culture composition and viability (Figure 5.2).

When assessing viability of cultures by quantifying the number of pyknotic nuclei, the genotype of MSN cultures contributed 10.13% of variation in a one-way ANOVA (p = 0.0216) however following Bonferroni correction for multiple comparisons, no differences were significant between control, or 75Q cells. Furthermore, conditioned media treatments did not cause any significant difference to culture viability regardless of HTT polyQ-length (p = 0.241 - 0.99) (Figure 5.2A).

The impact of conditioned media on MSN culture composition was also assessed by quantifying the percentages of βIII-tubulin+ and nestin+ cells within cultures (Figure 5.2B and C) using HCl. The percentages of βIII-tubulin+ cells did not differ between conditions when cells expressed non-HD HTT polyQ-lengths; overall genotype conferred 5.15% variation (p = 0.0755). However, when assessing the impact of CPN conditioned media on 75Q MSN cultures, significant differences were found (Figure 5.2B). A reduced percentage of neurons was found in cultures treated with fresh N2B27 media when compared to MSN control media (22.64%) although this result was not significant (p = 0.259). Incubation of 75Q cells with conditioned media from 22Q cortical cultures resulted in, on average, 35.86% more neurons than N2B27 media alone (p = 0.0072), similarly, 75Q cortical conditioned media also increased the neuronal percentage by 35.54% in comparison to N2B27 media alone (p = 0.0079).

Moving on to assessing the proportion of nestin+ cells in the cultures, which was assessed to identify if exposing MSN cultures to cortical conditioned media impacted the rate of differentiation and culture maturity, again a similar result was found in that control MSN cultures were not overtly affected by the differences in conditioned media composition (p > 0.99), however 75Q cells proved more susceptible to alterations (Figure 5.2C). Fresh N2B27 media resulted in cultures with on average 31.87%, 38.95% and 33.15% more nestin+ cells than MSN control media (p = 0.0075), 22Q cortical conditioned media (p = 0.0006) and 75Q cortical conditioned media (p = 0.0049). Although 45Q cortical conditioned media produced 15.57% more nestin+ cells than N2B27, this result was not considered significant (p > 0.99), nor were any significant differences found between MSN control media and cortical conditioned media (p > 0.99).
To quantitatively assess the levels of cell death in cultures, an LDH assay was run with the conditioned media samples after incubation was terminated. The caveat to this experiment is that LDH is stable within cell supernatant for 9 h, therefore as the incubation period of this experiment was much longer than this (48 h) the LDH value may be less accurate. In an attempt to normalise the values collected to minimize any variation from cell loss over time, LDH values were normalised to the total cell count provided by HCl, to give an LDH/cell (MSN) value, as presented in Figure 5. 2D. The media in which MSN-containing cultures were incubated caused 41.48% of the variation within the experiment (p = 0.0297), however once corrected for multiple comparisons, the only significant difference that remained was when 22Q cortical conditioned media was compared to N2B27 alone (+0.01474 LDH released in 22Q cortical conditioned media conditions, p = 0.0237). The discrepancy between cell death phenotypes is of interest and suggests that cell death by apoptosis or necrosis may differ in cultures, which will be discussed.

Taken together, these results suggest that 75Q MSN-containing cultures are more susceptible to alterations in culture conditions and media composition than those not expressing pathogenic HTT polyQ-repeat lengths, in terms of viability and impact on differentiation, thus may be more susceptible in co-culture conditions.
Figure 5. Conditioned media co-culture alters culture composition and viability of 75Q MSN-containing cultures. A conditioned media platform was set up to investigate the impact of CPN-released trophic factors on culture composition and viability of HD family series MSNs. The percentage viability (A), neuronal purity as measured by percentage βIII-tubulin+ cells (B) and nestin+ cells (C) was assessed, as well as the levels of LDH released per cell (D), were measured after incubation with MSN and CPN control medium (freshly supplemented MSN post-26 media and fresh N2B27 media) as well as conditioned media collected from d50 CPN-containing cultures of varying HTT polyQ-lengths. Data from 22Q and 30Q MSNs were collated and presented as control. Data are presented as mean ± SEM. (N=1, n = 3, n = 10) *, p < 0.05; **, p < 0.01; ***, p < 0.001.
5.5.3 MFC co-cultures

The use of human PSC-derived neurons to produce the CS pathway in MFCs has not yet been published, therefore the experiments to be discussed, and their findings, are novel contributions to the HD field.

As this project had worked most extensively with PSC derived cells from the HD family series, all experiments discussed from here on relate only to the HD family series, no IsoHD series co-culture data was collected. This decision was also influenced by the scale and complexity of completing these experiments, which precluded the use of many cell lines.

Although this project had identified PSC-derived CPN-containing cultures were able to survive and send axonal projections through MFC micro-channels, it was not clear whether PSC-derived MSN-containing cultures would be viable when plated into the axonal compartment of the device. Also, the optimal protocol for cell seeding (simultaneous, or sequential) had to be determined. Therefore a pilot experiment was set up; one 75Q cortical clone was seeded with either a 22Q striatal clone, producing a HD vs control culture (chimeric culture), or with a 75Q striatal clone, producing a HD vs HD culture. Two methods of plating were trialled. The first was sequential plating, whereby CPNs were seeded at a density of $4 \times 10^4$ cells into the somal chamber, allowed to attach for 30 min prior to flooding both somal and axonal chambers were N2B27 media to prevent evaporation. After 24 h incubation, the media from the axonal (MSN) chamber was aspirated and the same seeding procedure was completed, with MSN-containing cell suspension injected into the axonal chamber. It was immediately obvious that this was not a viable seeding protocol, as the MSN-containing cell suspension instantly 'flowed through' the chamber; the hydrostatic pressure in the device, plus remaining N2B27 media in the axonal chamber resulted in all cell bodies flowing directly through the chamber and collecting in the bottom well. The second plating method was simultaneous plating; although corticals were still the initial cell type seeded into the device, the injection of MSN cell bodies was immediately afterward. This protocol was found to be very effective and thus was the chosen seeding protocol for the remainder of the project.

The next parameter to be optimised was that of media changes, in their frequency and volume. Typically in routine culture, both PSC-derived CPNs and MSNs undergo half media changes every 48 h, however due to the small volume in which the cells are located it was tested as to whether increased media changing frequency would prolong cell viability. In fact, the opposite was found, half media changes every 24 h resulted in extensive cell detachment, which was hypothesised to be due to the shear stress that was caused as a result of media aspiration and replacement. Conducting half media changes every 48 h somewhat resolved cell detachment, however a higher level of debris was observed than anticipated. Indeed, when half media changes were conducted every 72 h, the health of the cultures appeared greatly improved. Moreover, it was observed that by maintaining MFCs in a horizontal position, and not tilting them to $45^\circ$ to encourage flow through by gravity, the integrity of the cultures was also
improved. For these reasons, half media changes were conducted every 72 h for the remaining experiments of the project.

One final parameter to assess was the longevity of the co-cultures; how long could the co-cultures be maintained such that the viability of the neurons was not compromised to an extent that experimental data was not reliable. This parameter proved challenging, as the longevity of the cultures differed between MFCs and appeared to correlate with the number of proliferating cells that persisted in culture. If a higher proportion of proliferating cells were in one or both of the chambers, the longevity of the cultures was much reduced; 72 h half media changes were not enough to sustain viability. In an attempt to counteract this caveat, the cells seeded into MFCs for co-culture experiments were always subject to the additional Accutase passage described in section 2.3.4 and 2.4.4 prior to plating.

Examples of the resultant co-cultures can be seen in Figure 5. 3. Brightfield images depict the axonal (MSN) chambers, where MSN cell bodies can be observed interacting with CPN axonal projections that have successfully emerged from MFC micro-channels. White arrows depict sites of neuronal process cross-over or interaction, which were hypothesised to be the sites of initial synapse formation. These co-cultures were maintained for 15 days prior to fixation and immunofluorescence staining to identify if CS synapses were indeed being formed. Once fixed, the cultures appeared robust enough to withstand the multiple washes required to reduce background signal levels, however as can been observed in the lower panels of Figure 5. 3, the immunofluorescence was not optimal, less so in HD vs control cultures than HD vs HD. Nevertheless, these experiments provided valuable insight into CS co-cultures within MFCs and formed the basis for the remaining experiments presented in this project.
Initial attempts at CS co-cultures plated in MFCs were carried out with 75Q CPN-containing cultures and either 22Q (left) or 75Q (right) MSN-containing cultures. Using brightfield microscopy on 40x magnification, interactions were visualised in the axonal chamber of MFCs, showing the interactions between CPN axonal projections and possible MSN cell bodies, as highlighted by white arrows. Co-cultures were maintained for 15 days prior to fixation and probing with axonal marker, tau, pre-synaptic marker, SYT1 and dendritic marker, MAP2. Example images sampled from the axonal chamber are shown, with zoomed in images of interaction sites shown in the lower panel. Scale bars represent 50 μm.

Figure 5. Initial MFC based co-culture system. Initial attempts at CS co-cultures plated in MFCs were carried out with 75Q CPN-containing cultures and either 22Q (left) or 75Q (right) MSN-containing cultures. Using brightfield microscopy on 40x magnification, interactions were visualised in the axonal chamber of MFCs, showing the interactions between CPN axonal projections and possible MSN cell bodies, as highlighted by white arrows. Co-cultures were maintained for 15 days prior to fixation and probing with axonal marker, tau, pre-synaptic marker, SYT1 and dendritic marker, MAP2. Example images sampled from the axonal chamber are shown, with zoomed in images of interaction sites shown in the lower panel. Scale bars represent 50 μm.
5.5.4 Chimeric MFC co-culture: pilot

Chimeric co-cultures were set up in which HD CPN-containing cultures (75Q clones 1 and 2) were co-cultured with control MSN-containing cultures (22Q clones 1 and 2). For each clonal combination, two co-culture MFCs were generated so that semi-longitudinal assessment could be conducted - the devices could be fixed at separate time points thus providing two ‘snapshots’ of sister culture integrity. Unfortunately, MFC devices containing 75Q (1) vs 22Q (2) became infected on day five and thus could not be used for experiments.

After ten days co-culture, one MFC combination was fixed, and immunofluorescent staining carried out to identify cells containing activated caspase-3 to give an indication of cell viability (apoptosis) and βIII-tubulin (Figure 5.4A). The majority of cells were neuronal and viable. As previous experiments had also indicated that co-cultures remained viable up to 15 days (section 5.5.3) the pilot experiment was extended.

The health of the cultures was monitored closely, using daily brightfield observation. After a further 13 days co-culture (d23 in total), it was observed that cultures were starting to deteriorate; an increase in dead cells and debris could be visualised in chambers, thus were fixed. Immunofluorescence was carried out targeting the pre-synaptic protein synaptophysin, and post-synaptic marker PSD-95, alongside MAP2, a dendritic marker. By acquiring Z-stacks of areas in which neuronal processes intersected it was possible to establish if pre- and post-synaptic markers were closely juxtaposed, and thus indicates the presence of a functional synapse (Figure 5.4B). To enable easier visualisation, maximum intensity projections of Z-stacks were magnified, as can be seen in the right panel of Figure 5.4B. The images demonstrated clear pre-synaptic and post-synaptic puncta (highlighted with white arrows), however the co-labelling of MAP2 appeared to confound visualisation of juxtaposition, therefore it was decided that future investigation would be restricted to synaptic proteins only.

It was concluded from these pilot experiments that CS co-cultures could be maintained in culture for three weeks prior to any compromise of cell viability, and that synapses could be identified within cultures, thus a full experiment was designed and completed as described below.
Figure 5.4. Immunofluorescence results from a pilot CS pathway co-culture experimental series in MFCs. PSCs from the HD family series were differentiated into CPNs and MSNs prior to seeding into MFC devices. Chimeric cultures were generated whereby HD CPNs were projecting onto control MSNs and maintained for either 10, or 23 days. A) CPNs (left panels) and MSNs (right panels) were fixed after 10 days co-culture and immunostained for activated caspase-3 (green) and neuronal marker βIII-tubulin (red) and nuclei (blue). B) MSNs fixed after 23 days co-culture were immunostained for a pre-synaptic marker, synaptophysin (green) and post-synaptic marker, PSD-95 (red) and nuclei (blue). Images were taken from the MSN chamber only (left panel), and zoomed images were also acquired. White arrows depict incidences of co-localisation. Scale bars represent 50 μm (A) and 20 μm (B).

5.5.5 Recapitulating the CS pathway in health and disease using MFCs

A comprehensive assessment of CS pathway connectivity and viability in health and disease was designed using PSC-derived neurons from the HD family series. This aimed to identify the effect of mHTT on viability and connectivity within the CS pathway by co-culturing MSNs and CPNs. By culturing neurons derived from non-HD PSCs and HD PSCs in different combinations within MFCs an assessment of the effect of genotype in each compartment could be carried out. Previous studies have indicated that the genotype of the pre-synaptic compartment is key to overall pathway health (Virlogeux et al., 2018). Each clonal system comprised two technical replicates (MFC devices) to enable repeated measuring longitudinally.

Twenty-four hours after simultaneous plating there was even plating distribution with a limited variability in cell density between clones. In some devices, cell-body clusters were observed, however the majority of cells attained single cell suspension. At the 24 h time point, all cultures appeared viable and attached, there was no evidence of micro-channel blockage and cells had adhered well to the substrate. Devices were monitored using brightfield microscopy for signs of infection or degeneration every 24 h, however all devices remained viable during the experimental timeline. The presence of persistently proliferating cells became evident in devices containing 22Q (2) MSNs in the axonal chamber however, therefore these co-cultures were excluded from quantitative analysis.

The first observational assessment of culture integrity was taken at d22 co-culture followed by assessments at the d30 time point. As can be seen in Figure 5. 5 and Figure 5. 6, a complex neural and interacting network was evident in both of the MFC chambers. Each co-culture combination will now be discussed, with the focus on morphology of CPN containing chambers when co-cultured with different MSN clones.

5.5.5.1 Characterisation of non-HD (22Q) CPNs within the pre-synaptic compartment

We aimed to assess the effect of the MSN chamber genotype within the post-synaptic compartment on CPN projection and network morphology. Non-HD (22Q) CPNs were plated into the presynaptic compartment and left to project onto MSNs derived from either non-HD (22Q) or HD (75Q) PSCs. Due to the preliminary data presented in Chapter 4, which showed a reduced CPN projection when treated with 75Q MSN conditioned media, it was expected that in co-cultures comprising HD (75Q) MSNs, a less complex network would be seen. Assessment of the CPNs by brightfield microscopy as presented in Figure 5. 5, showed that the morphology of CPN-containing cultures in terms of distribution and phase bright: dark ratio did not differ according to the post-synaptic MSN chamber genotype or clonal identity, which was
unexpected. It could be argued that CPNs within the 22Q CPN vs 22Q (2) MSN system exhibited a higher level of cell death (red asterisks), however as the MSN chamber was overrun by proliferating cells, resulting in the degeneration of that chamber, this observation was not surprising. This result was also seen in the second technical replicate (data not shown), suggesting the degeneration in the MSN chamber was indeed the driving factor in increased cell death in the CPN chamber. No overt morphological changes were noted in 22Q CPNs dependent on MSN chamber identity; cellular distribution appeared even throughout cultures and a high level of network complexity was observed irrespective of co-culture composition.

5.5.5.2 Characterisation of HD (75Q) CPNs within the pre-synaptic compartment

Following the assessment of non-HD CPNs as described above, the same assessment was applied to HD (75Q) CPNs when co-cultured with either non-HD (22Q) or HD (75Q) MSNs in the postsynaptic compartment. When assessing CPNs in these co-culture set-ups (shown in Figure 5.6), several interesting observations can be made. Firstly, that less cell death was observed in these cultures, as indicated by a reduced phase bright level. This was particularly obvious in cultures comprising 22Q (2) MSNs in the postsynaptic compartment, which contrasts the phenotype apparent in Figure 5.6. The cortical compartment remained viable with clear evidence of intra-neuronal interactions for both 75Q clones. It could be argued that a higher incidence of 'cabling' occurred when CPNs were cultured with 22Q (1) MSNs, this was particularly evident in 75Q (3) CPNs (red arrows). Surprisingly, in all co-cultures, a higher level of network complexity was observed in comparison to cultures where non-HD (22Q) CPNs projected onto MSNs (5.5.5.1).

5.5.5.3 Morphological comparison of MSNs

To assess if altering the genotype of CPN-containing cultures within the pre-synaptic compartment genotype had any overt effect on MSN chamber morphology or phenotype, brightfield images of each MSN chamber within a co-culture were collated for side-by-side comparison (Figure 5.7). These results are purely descriptive as only observational data was collected, therefore quantitative analysis did not take place.

In all co-culture combinations, evidence of ball and cabling was observed in 22Q (1) MSN-containing cultures; co-culture with 22Q CPN-containing cultures coincided with increased cell clustering within the MSN chamber (Figure 5.7, green box). The levels of phase-bright cells was also comparable between cultures (Figure 5.7, green and blue boxes).

Unfortunately it became apparent that 22Q (2) MSN-containing cultures comprised a high proportion of proliferating cells, which overran the cultures and resulted in widespread cell death as observed in the right panels of green and blue boxes in Figure 5.7. Overall, it was observed that MSN cultures appeared more neuronal i.e. contained a higher proportion of neuritic processes when co-cultured with 75Q CPNs (Figure 5.7 blue and red boxes). Within 75Q (1) MSN chambers, a clear difference was observed in the culture density and distribution (Figure 5.7, yellow and red boxes). When cultured with 22Q CPNs, 75Q (1) MSNs produced a much denser culture with a higher level of interaction (yellow box, left panel). Evidence of ball and
cabling was observed particularly when co-cultured with 75Q (1) CPNs, although this was not seen with 75Q (3) CPNs (red box). In fact, this co-culture produced the least dense MSN compartment, with the majority of visible cell bodies appearing phase bright. Finally, when comparing 75Q (3) MSNs with respective CPN compartments, subtle differences in phenotypes were observed. An elevated level of ball and cabling was observed when MSNs were cultured with 22Q (3) CPNs, and both MSN cultures when cultured with 75Q CPNs presented with a similar density (Figure 5. 7, blue box).

As the effects of CPN genotype on MSN morphology, distribution and cell death were not consistent across clones, the differences observed here may be random.
Figure 5. Brightfield images of 22Q CPNs projecting onto 22Q or 75Q MSNs after 22 days co-culture. Brightfield images of HD family series CPNs (22Q clone 3) and MSNs were acquired after 22 days of co-culture in MFCs. Images of both chambers plus micro-channels (left), CPN chamber alone (middle) and MSN chamber alone (right) show culture morphology and distribution. Each row of images depicts a different co-culture setup with 22Q (1), 22Q (2), 75Q (1) and 75Q (3) MSNs (top to bottom). Scale bars represent 100 μm (left panel) and 50 μm (middle and right panels). Red asterisks highlight areas with a high level of cell death.
Figure 5. Brightfield images of 75Q CPNs projecting onto 22Q or 75Q MSNs after 22 days co-culture. Brightfield images of HD family series CPNs (75Q clone 3) and MSNs were acquired after 22 days of co-culture in MFCs. Images of both chambers plus micro-channels (left), CPN chamber alone (middle) and MSN chamber alone (right) show culture morphology and distribution. Each row of images depicts a different co-culture setup with 22Q (1), 22Q (2), 75Q (1) and 75Q (3) MSNs (top to bottom). Scale bars represent 100 μm (left panel) and 50 μm (middle and right panels). Red arrows depict evidence of axonal cabling.

Co-culture  | CPN chamber  | MSN chamber
---|---|---

75Q CPN's co-cultured with:
- 20Q (1) MSN
- 22Q (2) MSN
- 75Q (1) MSN
- 75Q (3) MSN
Figure 5. Brightfield images taken of the MSN chamber in MFCs, at d22 of co-culture. A grid of MSN chamber images is shown to facilitate comparison of MSN culture gross morphology when co-cultured with different genotypes and clones of HD family series PSC-derived CPNs. Green = control vs control; yellow = control vs HD; blue = HD vs control; red = HD vs HD. Scale bars represent 50 μm.
5.5.5.4 Synapse formation in CS co-cultures at day 22

To confirm the formation of synapses between projected cortical axons and striatal cells in the axonal compartment of MFCs (where MSN-containing cells were seeded), neurons in MFCs were fixed at d22 and immunostained for a pre-synaptic marker, synaptophysin, and post-synaptic marker, PSD-95. Example images are presented in Figure 5. 8, and zoomed in images are also displayed in which pre- and post-synaptic puncta are clearly juxtaposed, indicating to the presence of a synapse. In order to quantify the distribution and frequency of synapses present in co-cultures and also identify any difference in the number of pre- or post-synaptic components, an ImageJ plug-in, PunctaAnalyzer was used (Ippolito and Eroglu, 2010). The results of automated batch analysis showed that numbers of pre-synaptic puncta were comparable across all co-cultures. In control vs control cultures, and HD vs HD cultures approximately ~600 pre-synaptic puncta were found. This was similar to when control CPNs synapsed with HD MSNs, however in HD vs control co-cultures an average of 872 pre-synaptic puncta per FOV was found (p = 0.282) However, when normalised to total number of puncta identified per FOV, no significant difference was found between cultures (p = 0.69) (Figure 5. 8A). Similarly, when assessing numbers of post-synaptic puncta, HD vs control co-cultures also had a higher number (average 1317 puncta vs 559 puncta in control vs control). Also, a trend was observed when comparing HD vs control, and HD vs HD cultures, with the latter presenting on average 626 fewer puncta (p = 0.0767). However, when normalised to total puncta per FOV, no significance was found (p = 0.69) (Figure 5. 8B). Finally, when assessing the percentage of co-localised synaptic puncta, no significant difference was observed between cultures (p = 0.98) (Figure 5. 8C).

5.5.5.5 Synapse formation in CS co-cultures at day 30

MFCs of each co-culture combination were fixed at d30 and subject to the same immunofluorescence analysis as described previously. The aim of these experiments was to identify if the formation of new synapses, and maintenance of established synapses was altered by genotype in the pre- or post-synaptic compartment. Example images of axonal chambers containing interacting neural processes can be seen in Figure 5. 9, with quantification of synaptic puncta as previously described in Figure 5. 9A - C. These data presented several interesting results, in that whilst control vs control co-cultures had 80% of the pre-synaptic puncta present at d22 (d22, 605 to d30, 485) all other co-culture combinations had a dramatically reduced percentage of pre-synaptic puncta (control vs HD = 61% loss, HD vs control = 65% loss, HD vs HD = 72% loss). When comparing the percentage of pre-synaptic puncta of total puncta within the d30 experiment, no significance was observed between cultures (p = 0.73) (Figure 5. 9A).

The result was similar for the normalised percentage of post-synaptic puncta (p = 0.71) (Figure 5. 9B). Interestingly however, in control vs control co-cultures, the numbers of post-synaptic puncta had actually doubled over time, from an average of 559 puncta at d22, to 1122 at d30. In contrast, all other co-culture combinations had lost post-synaptic puncta over time (control vs HD, 52% loss, HD vs control, 62% loss and HD vs HD, 49% loss).
Finally, the percentage of colocalised pre- and post-synaptic puncta i.e. synapses, was reduced in all co-cultures at d30 in comparison to d22 (Figure 5. 9C). Again, control vs control cultures showed the most limited reduction (36%) in contrast to control vs HD (74% loss), HD vs control (79% loss) and HD vs HD cultures (81% loss). Within the d30 experiment, no significance was found overall in a one-way ANOVA (p = 0.75) between conditions. Although the raw data suggests that over time, co-cultures that are comprised of either a pre-synaptic, or post-synaptic compartment containing pathogenic neurons, are compromised in their synaptic potential, the same results cannot be said upon data normalisation for total puncta count. It is important to note that these experiments were completed on sister cultures, thus it was not possible to distinguish if synapses were truly being lost, or if these cultures had a reduced number of synapses formed originally. Furthermore, as aforementioned, the loss of synapses over time could be attributable to higher levels of cell death observed within mHTT-expressing cultures. Finally, axonal vs dendritic synapses could not be delineated due to limitations of imaging; optimisation studies identified dendritic synapses by co-localisation with MAP2, however due to the strength of this signal, it was not possible to then identify single puncta, thus this antibody was removed from analysed experiments.
Figure 5.8. Immunofluorescence imaging of co-culture synapses and quantification after 22 days co-culture. HD family series PSC-derived CPN- and MSN-containing cultures were co-cultured in MFCs to recapitulate the CS pathway. Four co-culture conditions were generated, either with control CPNs projecting onto control MSNs (control vs control) or HD MSNs (control vs HD), or HD CPNs projecting onto control MSNs (HD vs control) or HD MSNs (HD vs HD). After 22 days co-culture, MFCs fixed and were incubated with antibodies targeting the pre-synaptic marker, synaptophysin (green), and post-synaptic marker, PSD-95 (red). Confocal images were acquired on 40x magnification (top panel) and zoomed images of synaptic interaction were selected (lower panel). Scale bars represent 10 μm. The percentage of pre-synaptic (A) and post-synaptic (B) puncta were quantified as a function of total puncta per FOV using automated analysis on ImageJ. The percentage of colocalised pre- and post-synaptic puncta (proxy readout for synapses) was calculated after applying a signal threshold and minimum separation distance, as a function of total puncta. Data is shown as mean ± SEM, (N=1, n = 2, n = 2 - 4 per condition, (22Q (2) MSN clone excluded)), a minimum of 3 FOV were obtained for each MFC. White arrows depict areas of co-localisation.
Figure 5.9. Immunofluorescence imaging of co-culture synapses and quantification after 30 days co-culture. HD family series PSC-derived CPNs and MSNs were co-cultured in MFCs to recapitulate the CS pathway. Four co-cultures conditions were generated, either with control CPNs projecting onto control MSNs (control vs control) or HD MSNs (control vs HD), or HD CPNs projecting onto control MSNs (HD vs control) or HD MSNs (HD vs HD). After 30 days co-culture, MFCs fixed and were incubated with antibodies targeting the pre-synaptic marker, synaptophysin (green), and post-synaptic marker, PSD-95 (red). Confocal images were acquired on 40x magnification (top panel) and zoomed images of synaptic interaction were selected (lower panel). Scale bars represent 10 μm. The percentage of pre-synaptic (A) and post-synaptic (B) puncta were quantified as a function of total puncta per FOV using automated analysis on ImageJ. The percentage of colocalised pre-and post-synaptic puncta (proxy readout for synapses) was calculated after applying a signal threshold and minimum separation distance, as a function of total puncta. Data is shown as mean ± SEM, (N=1, n = 2, n = 2 - 4 per condition (22Q (2) MSN clone excluded)), a minimum of 3 FOV were obtained for each MFC. White arrows depict areas of co-localisation.

5.5.5.6 Impact of pre-synaptic compartment on MSN viability

In an attempt to accurately quantify if pre-synaptic compartment genotype influenced the viability of MSN-containing cultures in the axonal chamber, the media from MFC chambers was collected and the ‘flow through’ media was subject to LDH analysis. Data was processed such that the viability of control MSNs i.e. 22Q, was compared when innervated by either non-HD (22Q) or HD (75Q) CPNs (Figure 5.10A). No significant difference was found between these conditions, because the variability in HD innervated MSNs was large. When comparing the levels of LDH released by HD MSNs innervated by HD CPNs (75Q) reduced the levels of LDH were released when compared to control innervated MSNs, however this result only trended towards significance (p = 0.0731) (Figure 5.10B). As such, there was no significant effect of presynaptic genotype on MSN viability. These experiments were somewhat limited due to the small sample number and available media volume, therefore interpretation is challenging.

Figure 5.10. LDH assay analysis of co-culture samples taken from MFCs at d30. The flow-through media aspirated from MFC co-cultures was run on an LDH assay at d30 co-culture and normalised to cell number. Levels of LDH released per A) 22Q MSN or B) 75Q MSN when the pre-synaptic compartment is either of control, or HD genotype. Data are presented as mean ± SEM (N=1, n = 2, n = 2 - 4 per condition (22Q (2) MSN clone excluded)).
5.6 Discussion

Models of the CS pathway have proven invaluable in developing our understanding of the pathology and degeneration that occur as a result of mHTT, and ultimately how diminished CS pathway connectivity contributes to HD pathogenesis and disease progression. Previous in vitro models of the CS pathway have been restricted to using primary neurons from HD rodent models (Kaufman et al., 2012; Milnerwood et al., 2012; Garcia-Munoz et al., 2015; Penrod et al., 2015; Buren et al., 2016; Virlogeux et al., 2018); to date there have been no published reports of an in vitro CS pathway co-culture model comprised entirely of human cells. The data presented here are therefore novel, and may be of benefit to the HD field.

Previous studies of the CS pathway in rodent co-culture models have suggested that CPN and MSN neuronal maturity and function is improved in a co-culture format, due to the increased activity and feedback each population receives (Buren et al., 2016). Furthermore, deficits in these features, amongst others, have been documented in co-cultures expressing mHTT (Buren et al., 2016; Virlogeux et al., 2018).

Several studies have focused on the electrophysiological differences of CS co-cultures. It was found that in YAC128 mice, extrasynaptic and whole cell NMDA receptor currents in HD MSNs were elevated. This was compounded by an increased expression of GluN2B subunits, which taken together, were hypothesised to contribute to elevated levels of MSN death in the mice due to excitotoxicity (Milnerwood et al., 2012). Virlogeux et al investigated the connectivity between CPNs and MSNs harvested from HdhCAG140/+ mice and co-cultured in MFCs. The authors observed that HD-HD (both CPN and MSNs were of mutant genotype) co-cultures exhibited a hyper-synchronised connectivity phenotype, with extended silent periods in which no firing was recorded from MSNs, followed by random, infrequent, short bursts of action potentials which were of 3-fold higher amplitude than WT cultures (Virlogeux et al., 2018). The authors were also able to investigate axonal trafficking by transfecting CPNs with mCherry-tagged BDNF, and tracking its movement in DCV along the axon. It was found that both anterograde and retrograde axonal trafficking was compromised in HD CPNs. Furthermore when assessing post-synaptic retrograde signalling in HD MSNs, the authors noted this function was also deficient in comparison to WT cultures. These studies indicated that, at least in rodent models of the CS pathway, the HD mutation confers a negative effect on both pre-synaptic and post-synaptic functionality in CS co-cultures, and as such, an investigation using human cells was warranted.

The preliminary experiments in this study, included directly mixed co-cultures and indirect interaction paradigms via the use of conditioned media. Experiments using a mixed population of PSC-derived MSN- and CPN-containing cultures were valuable in determining the gross viability of co-cultures when culture medium was shared. As connectivity was a primary area of interest for this project, the decision was made to seed both populations in the same culture device, thus enabling physical contact. In an attempt to more easily distinguish MSNs from CPNs within co-cultures, a GFP transfection was performed on MSN-containing cultures.
Despite this, no formal quantification was completed as although successful, the transfection efficiency was low, thus the sample size for quantification would not render meaningful results. Furthermore, it could not be validated if neurons were CPNs or simply MSNs that had not been successfully transfected. Although unsuccessful in this project, previous studies have utilised this technique (Kaufman et al., 2012; Milnerwood et al., 2012; Parsons et al., 2013; Buren et al., 2016; Buren et al., 2016), and gained insight into differences in connectivity as previously discussed. Other publications have employed a slightly different co-culture technique, in which coverslips seeded with either CPNs, or MSNs are then placed in a shared culture device, and flooded with a common media (Penrod et al., 2015). The authors focused on the morphological alterations that were caused as a result of MSN exposure to CPN trophic factors, and identified an elevated level of dendritic branching and arborisation suggesting an increased maturation rate.

In order to construct a more high-throughput experiment, to facilitate unbiased quantification, a conditioned media paradigm was employed in this project. By applying conditioned media from PSC-derived CPN-containing cultures with varying HTT polyQ-length, it was possible to examine any alterations in viability or culture composition as a result of the secreted trophic factors. It should be noted, that the constitution of CPN conditioned medium was not assessed, therefore any change to phenotype cannot be pinpointed to a particular growth or trophic factor. The results from these experiments suggested that whilst MSN-containing cultures with a non-pathogenic HTT polyQ length remained stable in terms of viability and culture composition, regardless of their external media, the same could not be said for 75Q cultures. This cohort had striking differences in culture composition both in terms of the neural purity and NSC content, however these differences were not HTT polyQ-length dependent. Indeed, whilst CPN unconditioned media (N2B27) and conditioned media from 45Q CPNs appeared to reduce the percentage of both NSCs and neurons within MSN-containing cultures, 22Q and 75Q CPN conditioned media led to a similar outcome in 75Q cells. A possible explanation for this could be due to the composition of the conditioned media; although samples from CPN cultures were taken at the same point in differentiation and processed identically, simple factors such as the density of CPN cultures could have affected the conditioning of the media; this was not controlled for due to error in experimental design and is thus a limiting factor. If cultures were sparser, fewer trophic factors may have been secreted, but on the other hand, metabolic components such as glucose may be increased due to a reduction in cell number. For these reasons, it is challenging to draw firm conclusions from these data. Moving forward, future experiments would require a higher level of control - CPN density would be matched at the time of seeding for conditioned media collection, and it would be necessary to analyse the components of conditioned media prior to application, by mass spectrometry for example. Alternatively, more simple questions could be asked, such as the effect of varying glucose, cytokine, or neurotrophin levels in MSN media. Despite this caveat, the data set proved valuable in identifying that a differential response to CPN conditioned media was observed in MSN-containing cultures, thus the next stage of experimentation was to introduce direct contact of CPNs and MSNs to assess any differences in CS pathway generation and connectivity.
Another interesting finding in this data set, was the contrasting results of cell viability when assessed by activated caspase-3 HCl or LDH assay. It is important to note that whilst both these measures can enable an estimation of culture viability, they do not have the same readouts. Activated-caspase 3 is a marker of cellular apoptosis primarily, plus the format in which it was assessed gave a 'snapshot' of the proportion of nuclei in a population undergoing nuclear condensation at that time. In contrast, LDH assays are indirect measures of the accumulated cell death that has occurred since the last full media change, and quantifies LDH released from damaged cells, including necrotic, ruptured or porous cells. This therefore could be an explanation for why the two measures of cell viability gave different results, particularly in the case of 75Q cells.

We aimed to generate a protocol in which the CS pathway and its connectivity could be modelled in vitro using human neurons. When designing a suitable platform, it was important to replicate the in vivo environment as far as technically and experimentally possible, although it is acknowledged that this recapitulation is much simplified in comparison to the brain. The data shown in section 5.5 illustrates the steps taken to design, optimise and generate a platform in which PSC-derived neurons could be co-cultured to replicate the CS pathway in MFCs, which were identified as an excellent tool due to the structural properties of the device and its easy manipulation. As aforementioned, the capacity of human PSC-derived neurons to grow in restrictive culture conditions was not known; data has been presented here to suggest that there was no hindrance on neuronal morphology or phenotype as a result.

Discussed now are a few ways in which the CS connection has been recapitulated by this model. The time at which CPN seeding occurred was a critical step in this experimental design, this is because, as already mentioned, the Shi et al protocol produces the distinct layers of the cortex at different times, thus the seeding of CPN-containing cell suspension was timed to coincide with the period in which layer V neurons were at peak emergence, as occurs in the brain during neurogenesis. The micro-channels within the MFC device are representative of the parenchyma that endogenous CPNs must project across in order to synapse with MSNs; in order to reach micro-channel entrances, CPNs may have had to migrate throughout the chamber also, depending on their final location once injected into the chamber, which is representative of CPN migration to the neural plate in the CNS. The model presented here is a true recapitulation of the CS pathway, as the formation of synapses were evident in MFC co-cultures, and whilst neuronal connectivity was not directly assessed per se, the fact that a substantial proportion of synapses were identified within an eight day time frame, suggests at least some level of connectivity; if no functional connectivity was present, the synapses would have degenerated in all likelihood.

On the other hand, there are many instances in which this model differs from the in vivo CS pathway. Within the brain, neurons are not in isolation, glial cells play a key role in not only establishing the CS pathway in terms of guiding CPN migration (Cooper, 2013), but also
maintaining and supporting synapses (Bacci et al., 1999; Lee and Chung, 2019). Furthermore, although the MFC devices were coated with laminin substrate, the extracellular matrix of the brain is much more complex (Novak and Kaye, 2000). Although the micro-channels simulate the need for axonal projection, in all likelihood, CPNs would have to project their axons much greater distances than 500 μm, although this has not been formally quantified previously in the literature. Finally, although steps were taken to try and allocate pre- and post-synaptic markers to CPNs and MSNs respectively, it is likely that auto-synaptogenesis was occurring to some degree i.e. MSNs synapses with themselves.

Completing co-culture experiments in MFCs is technically challenging, time consuming and costly, therefore to assess the feasibility of a full-scale study, and develop analysis methods, a pilot study was completed. The pilot study guided the design of the full experiment in terms of length of experiment, assessment measures, technical optimisation and analysis techniques. A power calculation was not completed, as cellular material at this stage of the project was extremely limited, thus the maximum number of experiments as feasible were completed.

The final experiment of this project was designed in such a way that a four-way comparison could be made. Although comparing the formation and functionality of an in vitro CS pathway in both health, and disease states was valuable, this project also wanted to investigate further, and attempt to tease out whether any dysfunction seen, could be more attributable to the pre-synaptic, or post-synaptic compartment i.e. whether the phenotype was cortical driven or MSN driven. For this reason, chimeric cultures were also constructed in which control CPNs projected onto HD MSNs, and vice versa. Importantly, a similar experimental design was carried out by Virlogeux et al and published in 2018. Therefore this was a great opportunity to compare HD phenotypes across species.

The quantitative data presented here showed that after three weeks of co-culture, no significant difference was found between either pure or chimeric cultures. During a time course experiment, it was found that total synapse numbers diminished with time in all cultures. At 30 days co-culture, control vs control cultures presented with a higher synaptic count than all other groups. The reduction in synaptic number over time may be due to diminished synapse maintenance in cultures that are expressing an expanded HTT polyQ-length in either cortical or striatal chamber, suggesting that regardless of the location of the expanded HTT polyQ, when present, a HD phenotype is observed. With that said, once data was normalised to total synaptic puncta count, no significant difference was found. This is in contrast to previous reports that are also contradictory with each other. Buren et al (2016) described an increased excitability of cultures was observed in WT-WT, WT-HD and HD-WT cultures, when assessed from d14 - d21 in co-culture, but was absent in HD-HD cultures, suggesting that mHTT expression is required both pre- and post-synaptically for a HD phenotype to emerge (Buren et al., 2016). In contrast, Virlogeux et al (2018) presented data showing that HD phenotypes were observed with equal severity in HD-WT and HD-HD cultures, whereas WT-HD cultures more
closely resembled WT-WT, suggesting that it is the genotype of the pre-synaptic compartment that drives CS phenotype and pathology (Virlogeux et al., 2018).

Whilst presenting contradictory evidence, both of these studies had the advantage of recording either electrophysiological data, or live imaging of glutamate release and/or calcium imaging. This adds an extra level of assessment as the functional connectivity of co-cultures could also be assessed. The issue of collecting quantitative functional data from within the paradigm presented here cannot be ignored. Due to the assembly of the MFC devices themselves, it was not possible to perform sophisticated electrophysiological recordings from these cultures such as paired patch clamp - stimulating a cortical neuron synaptically linked to an MSN which is then recorded from. Furthermore, whilst semi-quantitative measurements can be made using Fluo4 live dyes, the construction of the device combined with limited focal planes in confocal microscopy mean that data is less reliable.

With that said, there are several avenues of assessment which could be investigated for future studies. Park et al describes the use of reversible bonding in MFC assembly whereby the PDMS template can be removed from the glass coverslip. In principle, this would leave the co-culture and cellular connections intact thus rendering the culture amenable to patch clamp assessment. Furthermore, Garcia-Munoz et al. (2015) constructed a co-culture design in MEA whereby the neuronal populations were seeded on opposing sides of the array, further supported by a physical barrier which was maintained in place until recordings were taken. Using a transfection mediated route, such as transfecting neurons prior to co-culture with either of the vectors used in Virlogeux et al (iGluSnFR, or GCaMP6f), longitudinal assessment of culture activity and CS connectivity would be enabled. These are just a few of the options available for assessing the connectivity established between cortical neurons and MSNs in vitro.

Finally, in an attempt to address if co-culture with CPNs of either control or HD genotype impacted the viability of MSNs, LDH assays were run on samples collected from MFC co-cultures at the termination of the experiment. Analysis was completed by comparing the viability of control MSNs innervated by either control or HD CPNs, followed by the viability of HD MSNs innervated by control or HD CPNs. In the former, no significant difference was observed, indicating that in this experiment at least, the pre-synaptic compartment genotype did not impact on MSN-culture viability. In contrast, the latter comparison suggested that a lower level of LDH was released by HD MSN-cultures when innervated by the same HD genotype rather than control CPNs. This result was slightly surprising, as one would assume that CPNs not expressing mHTT may result in a more favourable culture environment, however this was not the case.

One possible explanation for this could be due to the genetic background and associated variability of the cells. HD vs HD cultures were in fact differentiated from the exact same starting material, whereas the control CPNs were of course sourced from another individual. Indeed, as the fibroblasts used for PSC-reprogramming were collected from individuals with vastly different ages (control = parent, HD = offspring) it is not unreasonable to suggest that the cells may
behave differently when co-cultured due to age-related changes. To overcome this caveat, future studies could replicate the above experiment in isogenic lines, so that genetic background is no longer a contributing factor.

Secondly, although quantitative, LDH assays measure total LDH released from necrotic, ruptured, or porous cells since the last full media change - this may not have been the most appropriate assay to use. Indeed it may have been more appropriate to quantify apoptotic and necrotic cell death distinctly using imaging techniques, or instead to quantify metabolic activity of the culture by MTT assay for example.

5.7 Limitations
The limitations described in Chapter 3 and Chapter 4 contribute to the limitation of this results chapter also. The inability to account for every cells' identity within both MSN- and CPN-cultures is compounded by the experiments described here, as there is a doubled probability that phenotypes observed from these co-cultures were in fact driven by non-neuronal, or unidentified neuronal populations. This is accepted as a limitation of this project and methods to overcome this caveat are discussed in the following chapter.

5.8 Summary
By completing a step-wise experimental series, exploring multiple avenues of assessment and optimising culture conditions, this project concluded that the CS pathway can be modelled in vitro using an entirely human system. This is the first evidence of the use of human PSC-derived neurons in MFCs and demonstrates that the devices enable the physical recapitulation of the CS pathway in the brain, and enable manipulation of pre- and post-synaptic genotypes. As such, the comparison of 'healthy' or 'diseased' pathways is possible, but also further investigation into the source of pathology i.e. pre-synaptic or post-synaptic drive, is possible. Although the phenotypes presented in this project were subtle, evidence was present of differences in culture phenotype depending on the disease state, suggesting that even at the earliest time point, the CS pathway and its activity may be altered in disease. For this reason, further study is warranted using the platform and protocols presented here.
Chapter 6 - Conclusions and future works

The CS pathway is known to have an integral role in the generation of movement and higher cognitive function, therefore when disrupted causes deficiencies and anomalies in these processes (Cepeda et al., 2007; Haber, 2016). Within HD pathogenesis, disruption to CS connectivity is understood to be an early event that leads to the development of a choreic movement disorder as well as cognitive function demise (Creus-Muncunill and Ehrlich, 2019). The pathway itself is composed of the two principally vulnerable neuronal populations that are the first to succumb to mHTT toxicity in the disease, as such, this is a highly investigated area of HD research. Most of this has been completed using HD animal models, due to the difficulty in sourcing and generating primary human cells for study. Here, data has been presented demonstrating the reliable generation of both PSC-derived MSN- and CPN-containing cultures, that exhibit robust HD-associated phenotypes, some of which are novel findings. Furthermore, for the first time, a human cellular model of the CS pathway has been generated in culture, that not only mimics many of the physical properties of the CS pathway in vivo, but has also shown initial signs of connectivity, with suggestions of HD-associated phenotypes emerging even in comparatively short culture times and at early developmental stages.

6.1 Conclusions and main findings

6.1.1 Characterisation and mHTT-dependent phenotypes in PSC-derived MSN-containing cultures

During HD progression, the MSNs of the striatum are the initial cells that are lost, as they are selectively vulnerable to the toxic effects conferred by mHTT (Bates et al., 2015; Cowan and Raymond, 2006). Studying this cell type on a cellular and molecular level in rodent models of HD has proven extremely informative, and as a result we have gained valuable insights into the pathology and dysfunction that is experienced by these cells during the course of the disease, such as hyperexcitability, reduced viability, and alterations to cellular morphology and subsequent function (Creus-Muncunill and Ehrlich, 2019). It cannot be overlooked however, that HD is a human disease, which does not occur in other species unless artificially induced. As such, it is of vital importance that cellular and molecular pathologies are studied in a human system, so that we can be confident that our findings translate well to the human condition.

The advent of iPSC technology, enables researchers to derive a variety of neuronal cell types from somatic tissue such as skin biopsies (Takahashi and Yamanaka, 2006). These can be sourced from healthy donors or disease patients and as such, we now have the ability to investigate human neuronal dysfunction in culture with the correct genetic background. This project utilised iPSCs derived from an HD family, in which the parent was unaffected and therefore acted as a hemi-isogenic control line, and three offspring, all of whom developed juvenile HD, and carried the mHTT gene with variable CAG-repeat length mutations. To further compliment these lines and corroborate findings, an isogenic allelic series was also used for experiments, to varying degrees of success (discussed below).
Using the established Arber et al protocol, MSN-containing cultures were produced from the HD family and IsoHD series PSCs and full characterisation successfully confirmed the likeness to true MSNs in the human striatum, including DARPP-32/CTIP2 expression, correct gross morphology and development of dendritic spines. However, a variety of subtle differences were identified with regard to culture composition, including a reduced proportion of neurons, reductions in the proportion of CTIP2 positive cells and of proliferating cells, as well as a reduced intensity of DARPP-32 signal compared with the non-HD control cultures. Over time, it was observed that MSN-containing cultures with the HD mutation experienced greater cell loss, however a larger proportion of the remaining cells expressed CTIP2, thus were considered more mature; although CTIP2 expression alone does not confirm MSN identity, it does confirm the cells are of LGE specification, and implies with extended culture the maturity into MSNs. This was accompanied by a reduction in the proportion of the culture that were NSCs and/or undergoing mitosis suggesting that it was in fact the precursor cells which were being lost over time. Indeed when viability was assessed using an LDH assay, at the earlier time points assessed a reduction in viability was observed in lines expressing an expanded HTT polyQ. However this result was reversed at later assessment time points, suggesting potentially that as a culture increases in maturity, it gains a level of robustness not common to cultures containing high proportions of immature cells.

As toxicity in the HD brain is high (Reddy and Shirendeb, 2012), it is hypothesised that the neurons may experience a high level of stress, which is further elevated either in acute forms when patients suffer from an infection for example, or chronically (Frank-Cannon et al., 2009). An attempt was made therefore to induce cellular stress in culture in vitro to replicate the environment of the brain. An acute withdrawal of the neurotrophic factor BDNF was completed, which was hypothesised to induce a stress response in PSC-derived MSNs; BDNF is key neurotrophin required for MSN survival and previous publications have reported success in inducing a stress-response upon BDNF withdrawal (Mattis et al., 2015; The HD iPSC Consortium, 2012). In both the HD family series and IsoHD series a reduction of viability in response to BDNF withdrawal was observed in lines expressing an expanded HTT polyQ-repeat. Although not tested here, it would be interesting to investigate if other cellular stressors that generate an unfavourable environment would result in the same response; manipulating culture conditions so they provide the types of stress neurons may experience in the HD brain, such as glutamate-induced toxicity, hypoxia or cytokine exposure, would confirm if the result seen here is universal, or simply in response to neurotrophin withdrawal.

As discussed previously (section 4.5), it is plausible that during the latter stages of neurodevelopment, MSNs may experience a deficient exposure to BDNF, due to a delayed or compromised formation of the CS pathway. Although not previously tested in the literature, this project designed a BDNF deficiency experiment in which PSC-derived MSN-containing cultures were never exposed to BDNF throughout the differentiation process, thus were BDNF deficient. These cultures were then compared to cultures that had undergone 4, 14 or 24 days BDNF exposure, the result of which was surprising. It was hypothesised that BDNF deficient culture would have a lower viability than BDNF treated cultures, and that over time, this loss of viability
would become more pronounced. This was not the case however. Instead, the impact of +/- BDNF, as well as genotype appeared to vary across the experimental timeline, suggesting the viability varied according to the developmental time point. Counter-intuitively, at the final assessment, when MSNs had been cultured with or without BDNF for 24 days, it was observed that regardless of the genotype, MSNs never exposed to BDNF had increased viability compared to those cultured with the neurotrophin. Furthermore, HD (75Q) MSNs had elevated viability above other genotypes, irrespective of BDNF treatment. Whilst challenging to interpret, it was hypothesised that the composition of the culture, and the way it may have been altered as a result of BDNF treatment or deficiency could have contributed to the result observed. It has been documented that NPCs are particularly vulnerable to the effect of BDNF withdrawal (Mattis et al., 2015), therefore it is possible that these cells could have been preferentially affected by BDNF deficiency resulting in their early degeneration. Consequently, as the proportion of NPCs within cultures decreased, the LDH released by cultures may also decrease thus resulting in data indicating a reduced level of cell death. As this project identified a reduced number of proliferating and nestin positive cells in 75Q cultures at d36 and d60, it could be argued that this threshold, so to speak, was reached sooner than controls cultures, thus generating the d50 result of a decreased cell death rate in 75Q MSNs. These are speculations however and further experimentation by way of longitudinal culture composition analysis in BDNF positive and negative contexts would be required to confirm this hypothesis, which could be achieved by co-staining with activated caspase-3 and ki-67/nestin/βIII-tubulin for example. Furthermore, it would be interesting to specifically look at the effect of mHTT and BDNF on NPCs alone, alongside neurons alone, which could be achieved by completing assessment at earlier time points, and using mitotic inhibitors, respectively.

The final series of experiments focused on the electrical properties of PSC-derived MSN membranes once matured. It was expected that differences in excitability would be observed as well as alterations to baseline membrane properties such as cell capacitance, as shown by previous publications (Conforti et al., 2018; HD iPSC Consortium et al., 2012; Nekrasov et al., 2016). This was not the case however, as no overt differences were found between genotypes of either the HD family series, or IsoHD series. It has been suggested by multiple groups that PSC-derived neurons are slow to mature compared to primary ex-vivo neurons in terms of neuronal electrical properties, due to the isolated culture conditions typically used that lack external innervation i.e. unless glutamatergic innervation of MSNs is present in the culture, the cells may not mature efficiently (Bardy et al., 2015; Penrod et al., 2015; Reddington et al., 2014; Xu et al., 2017a). If this was the case in these cultures - action potential events were admittedly relatively infrequent in these recordings - this could explain the lack of differential phenotypes in these cells as the mono-culture conditions used here did not provide glutamatergic innervation to MSNs. If recordings were to be repeated after a longer time in culture, or when cultures were provided external innervation or cultured in BrainPhys medium for example, it is possible that subtle differences in MSN membrane properties would emerge (Dr Gabriele Lignani, personal communication).
6.1.2 Optimisation, characterisation and mHTT-dependent phenotypes in iPSC-derived CPN-containing cultures

To date, there are only two published reports of PSC-derived CPNs with mHTT mutations (Conforti et al., 2018; Mehta et al., 2018), therefore this project was able to cast a wide net in terms of phenotyping, to unveil novel HD-associated phenotypes. To investigate the emergence of HD pathologies and phenotypes in the layer V CPNs, the same PSC series were differentiated using an adapted version of the Shi et al protocol. Adaptations were necessary due to a high level of cell death and culture failure that was experienced during the majority of differentiations midway through the project timeline. The delay caused by this unforeseen problem, combined with the extended culture time required to differentiate CPNs were the primary reasons as to why the majority of experiments were conducted with the HD family series only, complemented with an IsoHD control line, rather than the full IsoHD allelic series as originally planned. The adapted passage techniques used in this project are superior to those previously published, as demonstrated by the higher rate of culture survival and differentiation success. Furthermore, by altering the timings and frequency of passages, a higher degree of control was enabled for experimentation. With that said, several studies have been successfully published by using the original Shi et al protocol, so the susceptibility to premature death could be specific to the cell lines used here.

Unlike MSNs, there were fewer differences observed in the culture composition of CPN-containing cultures, with a subtle, but significant increase in the proportion of NSCs found in 75Q cultures, as well as a significant reduction in overall viability as assessed by both pyknotic nuclei as well as LDH release values across multiple clones. Within the HD family series, differences in the number of pre-synaptic puncta were observed between genotypes, however this did not correlate with differences in HTT polyQ-length.

Initial investigation into the morphology and behaviour of cultures led to a series of interesting experiments, resulting in novel findings. The adhesion capability of CPN-containing cultures was assessed at multiple time points during the differentiation process, and although adhesion was found to be decreased at the NPC stage, once layer V neurons began emerging this impairment was no longer apparent. This led to the investigation of alternative aspects of neuronal behaviour, such as the dynamic morphology of CPNs. Using longitudinal imaging, specific aspects of culture morphology and cell behaviour, such as neurite length and branching, as well as cell clustering over time, were assessed and distinct differences were observed between pathogenic and non-pathogenic lines. Similarly to MSNs, it was found that over time the number of cell body clusters actually reduced in 75Q CPN-containing cultures, in contrast to 22Q cultures. Combined with the reduced viability observed in these cultures also, it suggests that cell loss is occurring. The identity of these cells - mature neurons or NPCs - was not investigated therefore cannot be commented on. Furthermore, although no difference was found in the number of branch points produced by neurons, when normalised to the number of cell body clusters, a significant difference was observed between genotypes. This is most likely explained by the difference in cell body cluster number between 22Q and 75Q lines; as the
number of cell body clusters was significantly increased in 22Q cultures compared to 75Q, the division factor would be much greater resulting in a much smaller 'branches per cluster' value, thus leading to the significantly *increased* result found in 75Q cultures.

A novel investigation into axonal projection capability of these cultures within microfluidic devices identified an HTT polyQ-length dependent decrease in the number of axons projected over time. Also, the axonal networks generated post-projection were of very different complexities. It was observed that post-projection networks in lines expressing an expanded HTT polyQ had fewer interactions than control lines. This has important connotations for the formation of the CS pathway, as if correct and translated to human neurodevelopment, these experiments could suggest the very formation of the CS pathway is compromised in HD. This area of research is highly debated, as the majority of data suggests that HD patients do in fact have normal brain development, with normal structural properties and cortices that match the general population. However, some groups have identified a smaller overall brain volume in HD patients compared to controls, and imaging studies have showed reduced white matter thickness. Furthermore, these studies assess the macrostructure of the brain and thus do not assess the number of synapses for example in control and diseased states. As such, there is a debate still to be had as to the neurodevelopmental aspect of HD (Nopoulos et al., 2011; Paulsen et al., 2008; Tabrizi et al., 2011; Whitwell et al., 2001). It would have been possible to repeat these experiments focusing on MSN projection also, however the decision was made to focus on CPN axonal projection as it was believed that it would prove more insightful in the context of the CS pathway; although MSNs are projection neurons also, the downstream projections of MSNs were not the primary focus of this project. In future studies, it would be interesting however to assess if MSNs followed a similar pattern to the CPN data presented here i.e. an HTT polyQ-length dependent decrease in axonal projection ability.

Finally, an important aspect of CS pathway biology, the role of BDNF was assessed in PSC-derived CPN-containing cultures in terms of production and release – reported to be altered in HD. High levels of experimental variability confounded clear interpretation of results but trends suggested both a reduction in the production of BDNF protein, as well as reduced BDNF release in lines expressing an expanded HTT polyQ. One aspect of these experiments which was surprising, was the limited effect of both stimulatory agents KCL and glutamate on BDNF release on these cells. These agents were chosen as they are known to stimulate activity-dependent release of proteins, including BDNF from neurons. However, neither agent appeared to significantly increase the probability of release. As both agents were correctly formulated with new reagent materials, it seems unlikely that this can be explained due to inactivity of the compounds. More likely, is that the cellular targets of these compounds, i.e. ion channels, were not present in sufficient quantity or density to elicit a biological response. Indeed, whilst indications of activity had been confirmed in these lines at a much later time point, there was no investigation into the expression levels of ion channels or transmembrane receptors at the time point at which experiments were conducted, which may have provided an explanation for this phenomenon.
6.1.3 Generation of a co-culture platform that accurately models the corticostriatal pathway

The major aim of this Thesis project was to generate a platform in which the CS pathway could be modelled *in vitro* using human cells. The data presented here demonstrates this is entirely possible and as such, this project was successful. The ability to construct a human CS pathway *in vitro* opens the door for studying multiple aspects of CS connectivity and early events in HD pathogenesis, which were previously impossible. Thus, the generation of this platform provides a key starting point for ongoing investigations into CS connectivity disturbance, and pathology in HD, in a human context.

As not previously used with human PSC-derived cells, the optimisation process necessary for creating a human PSC-derived neuronal co-culture system in MFCs was lengthy, but technical aspects were reproduced to an extent from those used for murine primary neuronal systems (advice was provided by Prof Schiavo lab members). The optimal density for cell seeding as well as seeding technique required several rounds of improvement, which combined with the length of time required to generate cellular material, contributed to the time-consuming nature of this project. As such, it was only possible to complete one full round of CS co-culture experimentation but much ‘proof of principle’ data was collated.

Nevertheless, measures of connectivity were possible, such as the formation of synapses, and an interesting result was found in terms of the number of synapses formed at distinct time points in co-culture. It was found that in HD vs control co-cultures (HD CPNs projecting onto control MSNs), an elevated number of synapses were present after three weeks of co-culture, confirming a higher level of connectivity between neuronal populations, contrary to what might be predicted. As aforementioned, this could lead to a hyperexcitable phenotype, however confirmation of this is technically difficult and outside the scope of this project. It could be argued however, that the most interesting observation was that the numbers of synapses identified in cultures were decreased over time whenever *either* the pre-synaptic or post-synaptic compartment contained HD neurons. In contrast, when cultures were comprised of all control neurons the degree of synapse loss was much reduced. As these findings are in contrast to those previously published in rodent models of the disease, where one study found the pre-synaptic compartment genotype only to determine phenotype (Virlogeux et al., 2018), and another study in which mHTT expression was required in both pre- and post-synaptic compartments to result in dysfunction (Buren et al., 2016), the importance of studying phenotype presentation in a human species model is emphasised. Possible explanations, aside from model species, that could account for the differences in results presented here include: polyQ-repeat length - both the published papers used cell lines with substantially higher polyQ lengths than those used here (128Q and 140Q vs 75Q); the readouts also differed, as Buren *et al* analysed striatal excitability, and Virlogeux *et al* assessed glutamate transmission and ERK signalling; finally the experimental timelines of the studies also differed - both studies used primary neurons, and completed assessments at d14, and d21 (Buren only), however it is acknowledged that primary neurons mature at a much faster rate than human PSC-derived
neurons, thus the difference in pre- and post-synaptic compartment contribution to phenotype may be development time-point dependent. As discussed in the following section, to validate the results presented here, the replication of these experiments in additional human cell models is of paramount importance.

6.2 Future works

6.2.1 Cellular composition analysis
As discussed within each results chapter of this Thesis, the cellular composition of cultures (both MSN- and CPN-containing) was not fully elucidated, and therefore limited the interpretation of significant results. To continue this work, investigations must first be completed into revealing the identity of unknown cells within both MSN and CPN cultures. There are several methods that could achieve this, that range in their complexity and predicted success. Increasing the panel of immunolabelling within PSC-derived cultures could contribute to revealing more cell types present, however the caveat of this approach is that antibody reliability must be high in order to be confident of the results. An alternative method to ensure a more homogenous culture of either MSNs or CPNs could be to purify the cultures via cell sorting. One method that could accomplish this is fluorescence-activated cell sorting (FACS), which can separate a heterogenous culture into distinct cellular populations dependent on their fluorescent labelling. This technique was used alongside this Thesis in an attempt to separate direct and indirect pathway MSNs derived from PSCs, however resulted in a very low efficiency rate (~5%) and caused widespread cell death due to the harshness of the technique. As such, further investigation would have to be conducted to identify a method by which a pure homogenous MSN and CPN culture could be derived from the heterogenous PSC-derived cultures presented here.

6.2.2 Dendritic morphological comparison
Within the scope of this project, confirmation of the presence of dendritic spines was carried out, demonstrating robust formation of mature ‘mushroom’ spine heads. The technique used, super resolution microscopy, enables a high level of detail to be resolved. However, a resolution: time trade-off is profound; high resolution imaging requires an extended image capture time, and post-acquisition processing is laborious. Therefore this technique, although extremely informative, is low-throughout, alas it is not suitable for large scale studies. Previous publications have reported success using Sholl analysis in both in vivo (Lerner et al., 2012; Indersmitten et al., 2015; Buren et al., 2016) and in vitro systems (Penrod et al., 2015). Moving forward, in optimal culture density conditions it may be possible to combine HCI with post-acquisition Sholl analysis (http://fiji.sc/Sholl_Analysis). This would enable the robust comparison of dendritic branching and arborisation between control and HD cultures. Furthermore, by utilising the IsoHD line, genetic background heterogeneity would be avoided and an HTT polyQ-length dependent effect could be resolved. Alternatively, although not successful in initial attempts here, using antibodies targeted to proteins enriched in dendritic spines such as spinophilin could also be pursued and used in a high content format. Spinophilin staining was attempted in this project, however the strength of signal achieved even after antibody
optimisation was not sufficient to enable quantification, thus alternative antibodies must be
trialled. The Harmony imaging analysis software used throughout this project is compatible with
add-on software, such as PhenoLOGIC (Perkin Elmer). This plug-in enables machine learning
to develop an analysis algorithm that can be applied to samples in batch processing. The
algorithm is developed using a ‘learn-by-example’ approach, whereby the user completes a
series of ‘training runs’ on representative images, specifying the upper and lower limits for object
parameters such as size, texture and morphology, which lends itself perfectly to the
identification of dendritic spines.

6.2.3 BDNF trafficking and post-synaptic uptake
A running theme of this project was the role of BDNF in the CS system: its production,
processing and release from PSC-derived CPN-containing cultures, and the impact of BDNF
withdrawal or deficiency on MSN-culture viability were both key areas of investigation. What
was not investigated however, was the axonal trafficking of this protein in DCVs, due to a
limitation of resources and inability to acquire appropriate constructs. Summarised are just a
few reports that document deficient BDNF production, trafficking and release, and suggest that
it may contribute to the pathology observed in HD. Drouet et al revealed that mHTT confers a
dominant negative effect over WT function; WT HTT facilitates vesicular trafficking, of which
BDNF is cargo, however the presence of a single mutant allele significantly reduces vesicular
transport, which can then be restored by selectively silencing the mutant allele only (Drouet et
al., 2014). Several publications have documented a reduction of BDNF protein in HD models,
including both animal models (Gauthier et al., 2004; Apostol et al., 2008; Baydyuk and Xu,
2014) and human tissue (Ferrer et al., 2000), which appear to correlate with disease
progression, however there is contrasting evidence around this matter with regard to specific
brain regions. Zuccato and Cattaneo identified in 2007 that cortical mRNA and protein levels of
BDNF were reduced in post-mortem tissues collected from 20 HD patients as early as the pre-
symptomatic phase. In contrast, Ferrer et al reported no significant difference in protein levels
in the parietal and temporal cortex, yet decreased protein in caudate and putamen, which was
also supported by other groups (Gauthier et al., 2004). The protocol used to process samples,
or even the detection methods used may account for these discrepancies in results, however as
animal model data as well as PSC-derived CPN data presented here and elsewhere suggest
that BDNF production and expression is lowered in HD, it is plausible that this is a true
phenotype. Regarding BDNF trafficking which unfortunately could not be assessed here, there
is a huge array of evidence demonstrating reduced volume and reduced velocity of trafficking in
both the anterograde and retrograde direction within HD (Baydyuk and Xu, 2014;
Nithianantharajah and Hannan, 2013; Raymond et al., 2011). Virlogeux et al identified that there
was both a reduction in the number of secretory vesicles produced in HD animal models, as
well as a reduction in the velocity of anterograde and retrograde transport. The mechanism
behind reduced velocity has been well characterised; it has been suggested that mHTT reduces
the efficiency of vesicle transport due to its interaction with the microtubule-based motor
complex. Accumulation of mHTT results in increased binding affinity to the p150\(^{glued}\) subunit of
dynactin, and its interaction partner HAP1. This results in a reduced velocity of vesicle trafficking along microtubules, hence BDNF trafficking is deficient in HD (Gauthier et al., 2004).

A future direction of this project could be to ascertain if any differences in axonal trafficking are present in both the HD family series and IsoHD series, dependent on HTT polyQ-length. As BDNF is particularly pertinent to this project and the wider context of HD as a disease, assessing the trafficking of DCVs along axonal tracts would be valuable. Previous publications have used tagged-BDNF and live imaging experiments to assess axonal transport (Adachi, Kohara and Tsumoto, 2005), however if combined with MFCs, these experiments could have added value (Zhao et al., 2016, 2014). The analyses of trafficking experiments is challenging, regardless of whether the cargo is for example, vesicles or mitochondria. The most common method used is KymoAnalyzer, a software which automatically tracks moving objects from time-lapse live imaging data (Neumann et al., 2017). Whilst incredibly useful, one of the caveats of this software is that directionality must be set in order to distinguish between anterograde and retrograde transport. In standard cell culture vessels, neurons are not limited in their growth direction, creating a complex network of interacting processes, which would of course, not be amenable to KymoAnalysis, unless extremely low-density cultures could be assessed; PSC-derived neuronal cultures do not respond well when cultured in relative isolation. One of the intrinsic properties of MFCs however, is the introduction of neural projection directionality. By culturing PSC-derived neuronal cultures transfected with either quantum-dot labelled BDNF (Zhao et al., 2014), or fluorescently tagged BDNF (Zhao et al., 2016) in isolation (CPN monocultures) or a CS co-culture context in MFCs, it would be possible not only to assess the anterograde and retrograde trafficking of BDNF in CPNs, but also the transmission and uptake of BDNF post-synaptically, as well as the retrograde transport once internalised in MSNs. As such, the trafficking of BDNF in its entirety could be assessed. Following a similar design to experiments completed in the Saudou laboratory, the trafficking of BDNF could be assessed in both pure, and chimeric cultures (Ehinger et al., 2019; Virlogeux et al., 2018), to identify the impact of compartment genotype on culture phenotype.

6.2.4 IsoHD series co-cultures in MFCs

The data presented here was the first documentation of a human CS pathway being replicated in vitro using PSC-derived neurons. Whilst successful with regard to pathway generation and the indication of HD-associated phenotypes, factors were present which could have confounded the results. In order to have more confidence in the data generated, it would be valuable to replicate the study using the IsoHD allelic series presented here, as originally planned. The use of neurons with identical genetic backgrounds would re-assure the field that any phenotypes observed were in fact a direct result of the expanded HTT polyQ-length, and not due to genetic heterogeneity. Furthermore, as the IsoHD series comprises a varied CAG-repeat length, the effect of increasing HTT polyQ could be investigated with regard to CS pathway formation and connectivity.

To add a further element of investigation, once the baseline phenotypes of pure, and chimeric CS co-cultures are established in MFCs, it would be possible to introduce treatments to these
cultures and record any alteration to phenotype. Although to date there are no disease modifying treatments for HD, antisense oligonucleotide (ASO)-mediated HTT lowering is in late-stage clinical trials, with promising results with regard to safety and efficacy, as well as quantitative mHTT concentration lowering so far reported at all stages (Tabrizi et al., 2019). The function and impact of these ASOs on the cell models presented here are currently being tested in the Tabrizi laboratory, therefore if preliminary evidence is collected that supports HTT-lowering in vitro, it would be interesting to incorporate this treatment option into future study design. For example, by application to either pre- or post-synaptic compartment neurons in isolation, it could be assessed if a reduction in HTT expression even after formation of the CS pathway, could reverse any pathogenic phenotypes observed. The limitation of these devices however, is that experiments can currently only be conducted in low-throughput, which may hinder the range of experimental questions that could be asked.

6.2.5 Construction of the CS pathway using tri-culture

Cellular co-culture methods that recapitulate brain pathways, including the CS pathway, have numerous advantages over rodent cell models or animal model brain slices. However, in order to truly recapitulate the human CS pathway, a much more complex co-culturing system would be required, that incorporated the presence of a multitude of support cells as found in the brain such as astrocytes and microglia. Understandably, these studies have not yet been completed, most likely due to the incredibly complex culture system that would be required. Due to the advent of more sophisticated microfluidic devices however, the idea of a tri-culture system is now within reach. The MFC devices used in Virlogeux et al have a third chamber incorporated into the design (see publication for diagram). The authors only assessed synapse formation within this central chamber in an attempt to assess true cortical-striatal synapses. This format could be redesigned however to include astrocytes within the central chamber. Within the field of neuroscience, the concept of a tripartite synapse is widely accepted, suggesting that true synapses are not readily formed or maintained without the support of adjacent astrocytic end feet (Araque et al., 1999; Perea et al., 2009). Furthermore, protocols for the differentiation of PSCs to astrocytes have now been developed and are beginning to be optimized by research groups (Krencik and Zhang, 2011; Shaltouki et al., 2013; Tcw et al., 2017) (Gordon CAG Triplet Repeat Conference, Tuscany, 2019). Thus, if CPNs, MSNs and astrocytes could all be derived from the same PSCs and co-cultured in tri-chamber MFCs, it is plausible that tripartite synapses could be observed for the first time in vitro. Tri-cultures have been completed before, with astrocytes, neurons and microglia tri-cultured in MFCs to study Alzheimer's disease (Park et al., 2018), as well as models of the blood brain barrier designed with astrocytes, endothelial cells and pericytes (Thomsen et al., 2015). This project could not find any evidence of tri-culture systems in the literature, which targeted the recapitulation of the CS pathway in HD. Coincidentally, through collaborations at UCL, tri-chamber MFC templates similar to the design described by Virlogeux et al are available for use. Furthermore, members of the Tabrizi laboratory are currently trialling HD family series and IsoHD series differentiation into astrocytes. It is therefore entirely plausible, that with intensive optimisation, this project could go on to develop the first-in-human CS pathway tri-culture.
To take this one step further, and enable repeated and non-invasive electrical recordings from cultures, it would be possible to bond the tri-chambers to custom designed MEAs, similar to Garcia-Munoz et al., (2015). In this context, recordings from distinct electrodes would be enabled, plus with the ability to switch MEA electrodes from ‘recording’ to ‘stimulating’, current could be applied to a specific chamber within the tri-culture - the CPN chamber for example - whilst simultaneously recording from the synaptic/astrocytic and MSN chamber. It would therefore be possible, to assess location specific activity, without the need for transfection or interference of the cells.

Similarly, if established, this tri-culture system could be used as a secondary screen for therapeutics. Once fully optimised and characterised at baseline, the addition of selected therapeutic compounds, such as ASO’s, to distinct chambers would be possible, the results of which could then be compared to baseline data to assess any improvement or detriment to phenotype. It should be noted that these experiments would not be high-throughput due to the nature of culturing within MFCs, therefore could not be used as a primary therapeutic screen.

In conclusion, this project has successfully created a co-culture system that is capable of modelling the CS pathway, in both an unaffected and HD format with human cells. The expansive phenotyping and characterisation of PSC-derived MSN- and CPN-containing cultures with CAG-repeats ranging from 22Q - 81Q has complemented previous studies in the literature, as well as added additional phenotypes that are novel to the field. With a wide range of future avenues of investigation open, this thesis has generated a platform from which future study can be built.
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