NEUROPROTECTIVE EFFECT OF SIGMA-1 RECEPTOR AND ACTIVIN A ON SYNAPTIC
FUNCTION AND CALCIUM HANDLING IN HUNTINGTON DISEASE

by

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Abstract

Huntington disease (HD) is a monogenic disorder with autosomal dominant inheritance. In HD patients, neurons in the striatum and cortex degenerate, leading to motor and cognitive disorders. Dysregulation of synaptic function and calcium handling is common in many neurodegenerative diseases. N-methyl-D-aspartate (NMDA) receptor function is enhanced at extrasynaptic sites, altering the balance of calcium-dependent neuronal survival vs. death signalling pathways. Another important level of calcium regulation is at the endoplasmic reticulum (ER), and this regulation is abnormal in HD. The ER is also suggested to be involved in nuclear calcium signalling, and I hypothesise that this signalling pathway is altered in HD. Sigma-1 receptors (S1Rs) – proteins located on the ER – play an important role in calcium regulation and thus gene transcription. Interestingly, activating S1Rs has been shown to normalise this ER calcium handling and restore synaptic function in HD mouse models. Furthermore, recent work has shown that overexpression of Activin A, a secreted protein whose transcription is nuclear-calcium-dependent, reduces toxic extrasynaptic NMDA receptor signalling in the hippocampus. The goal of this project is to determine the link between S1Rs, calcium handling, Activin A, and synaptic function to better understand the pathophysiological mechanisms of HD and to find new potential treatments. Neuronal cultures, imaging techniques, behavioural assessment, and electrophysiology were used to investigate these processes in a mouse model of HD. Our data shows contributions of different calcium channels to nuclear calcium signalling. Calcium imaging also suggests impairments in nuclear calcium signalling in HD striatal medium-sized spiny neurons in co-culture with cortical neurons, which was not corrected by treatment with S1R agonists. Furthermore, our data show that Activin A is decreased in HD culture media, and its overexpression normalises extrasynaptic NMDA receptor expression. Moreover, early injection of
an Activin A AAV virus into the striatum led to a significant improvement in a motor coordination task at an age when HD mice are known to show impairment. This project has elucidated therapeutic benefits of Activin A in the treatment of HD; more research is needed to understand Activin A’s mechanism of action and further explore its potential benefits in other neurodegenerative diseases.
Lay Summary

Huntington disease (HD) is a devastating inherited disease that can be detected before symptoms arise, but which is incurable. In HD patients, neurons involved primarily in motor functions degenerate, leading to motor and cognitive problems. Neurons, which transmit signals in our brains, rely heavily on calcium for their function. Dysregulation of the function of the connections between neurons (synaptic function), and their ability to regulate calcium levels, is a common characteristic of many neurodegenerative disorders such as Parkinson’s, Alzheimer’s, and HD. In this thesis, I found that mice that carry the HD genetic mutation have altered calcium signalling. The neurons of these mice also have decreased levels of a neuroprotective protein called Activin A, and that increasing its expression improves the performance of these mice on a motor task. Given the lack of current effective therapies for HD patients, this research presents Activin A as a new therapeutic avenue.
Preface

All experiments in this thesis were carried out in accordance with the Canadian Council on Animal Care and approved by the University of British Columbia Committee on Animal Care (certificate numbers A17-0295 and A19-0076).

The work in Chapter 2 has not been published. I designed the experiments with help from Dr. Lynn Raymond. The python code used for automatic placement of regions of interest (ROIs) using machine learning, calculation of ΔF/F0, and extraction of trace parameters such as timing, frequency, and amplitude of calcium peaks, as well as total area under the curve (AUC) of these peaks was written by Peter Hogg from the Haas lab. The code can be found in the appendix section. At the end of the thesis. Except for assistance from Judy Cheng who contributed data on Figure 2.5D-F, I performed all the experiments and analysed all the data. A manuscript is being prepared for publication.

The work in Chapter 3 has not been published. I designed the experiments with help from Dr. Lynn Raymond. The Activin A ELISA experiments in Figure 3.1 and 3.2 were performed by Jean Oh, and analysed in collaboration with Jean Oh. The GluN2B surface/internal experiment in Figure 3.4 was performed by Dr. James Mackay, and analysed in collaboration with Dr. Mackay. I performed and analysed data for all other experiments.

The work in Chapter 4 has not been published. I designed the experiments with help from Dr. Lynn Raymond. Lily Zhang, Pumin Wang, and I performed the mouse surgeries. The rotarod data in Figure 4.1 was performed and analysed by Daniel Ramandi. I performed electrophysiological experiments in Figures 4.2 and 4.3 in collaboration with Dr. Marja Sepers.
and Daniel Ramandi. I analysed the data of Figures 4.2 and 4.3. A manuscript with data from Chapters 3 and 4 is being prepared for publication.
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<th>Definition</th>
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<tbody>
<tr>
<td>2APB</td>
<td>2-Aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>3-PPP</td>
<td>N-n-propyl-3-(3-hydroxyphenyl) piperidine</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACVR</td>
<td>Activin receptor</td>
</tr>
<tr>
<td>ACVR1B</td>
<td>Activin receptor 1B</td>
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<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
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<td>ALK4</td>
<td>Activin-like kinase 4</td>
</tr>
<tr>
<td>AP-2</td>
<td>Adaptor protein</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BACHD</td>
<td>a full-length transgenic mouse model of HD</td>
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<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>a mouse genetic background</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CaV</td>
<td>Voltage gated calcium channel</td>
</tr>
<tr>
<td>CaV1.1</td>
<td>L-type voltage gated calcium channel</td>
</tr>
<tr>
<td>CBA</td>
<td>a mouse genetic background</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element binding protein</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>D-APV</td>
<td>D-2-Amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>DHPG</td>
<td>3,5-dihydroxyphenylglycine</td>
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<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>EF3</td>
<td>Elongation factor 3</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506-binding protein</td>
</tr>
<tr>
<td>FVB/N</td>
<td>an albino inbred mouse strain</td>
</tr>
<tr>
<td>Fyn</td>
<td>a tyrosine kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCaMP</td>
<td>Green genetically encoded calcium indicator</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GluN</td>
<td>NMDA receptor subunit</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>HAP1</td>
<td>Huntingtin-associated protein 1</td>
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<tr>
<td>HD</td>
<td>Huntington disease</td>
</tr>
<tr>
<td>HdHQ150</td>
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<tr>
<td>HEK293t</td>
<td>Human cell line</td>
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<tr>
<td>HTT</td>
<td>Huntingtin protein</td>
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</table>
Hz  hertz (1/s)

IONIS-HTT<sub>Rx</sub> ASO targeting HTT RNA

IP3  inositol (1,4,5)-trisphosphate

IP3R1  type 1 inositol (1,4,5)-trisphosphate receptor

IP3R2  type 2 inositol (1,4,5)-trisphosphate receptor

IP3R3  type 3 inositol (1,4,5)-trisphosphate receptor

Jacob  Juxtasynaptic attractor of caldendrin on dendritic boutons protein

KD  Knock-down

kDa  kilodalton

KI  Knock-in

KIF5C  Dynactin and the kinesin family member 5C

MAPK  Mitogen-activated protein kinases

mGluR1/5  Metabotropic glutamate receptor type ½

MK-801  Dizocilpine

MSN  Medium spiny neurons

mTOR  mammalian target of rapamycin

N171-82Q  a fragment transgenic mouse model of HD

NES  Nuclear export signal

NF-kB  Nuclear factor-kB

NMDA  N-methyl-D-aspartate

p53  Tumour-suppressor protein 53

PBS  Phosphate buffered saline

PFA  Paraformaldehyde
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>polyQ</td>
<td>Polyglutamine</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PRD</td>
<td>Proline-rich domain</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>PTKR</td>
<td>Protein tyrosine kinase-linked receptor</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
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<td>R6/1</td>
<td>a fragment transgenic mouse model of HD</td>
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<tr>
<td>R6/2</td>
<td>a fragment transgenic mouse model of HD</td>
</tr>
<tr>
<td>RCaMP</td>
<td>Red genetically encoded calcium indicator</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>S1480</td>
<td>Serine 1480 residue</td>
</tr>
<tr>
<td>S1R</td>
<td>Sigma-1 receptor</td>
</tr>
<tr>
<td>sEPSCs</td>
<td>Spontaneous excitatory postsynaptic current</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco-endoplasmic reticulum calcium ATPase pump</td>
</tr>
<tr>
<td>SGK</td>
<td>Serum- and glucocorticoid-induced kinase</td>
</tr>
<tr>
<td>SNARE</td>
<td>v-Soluble NSF Attachment Protein Receptor</td>
</tr>
<tr>
<td>SOCR</td>
<td>Store-operated calcium response</td>
</tr>
<tr>
<td>SP1</td>
<td>Specificity protein-1</td>
</tr>
<tr>
<td>STEP</td>
<td>Striatal-enriched protein tyrosine phosphatase</td>
</tr>
<tr>
<td>STIM</td>
<td>Stromal interaction molecule</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TMS</td>
<td>Total motor score</td>
</tr>
<tr>
<td>TRalpha1</td>
<td>Thyroid hormone receptor-alpha 1</td>
</tr>
<tr>
<td>UHDRS</td>
<td>Unified Huntington's Disease Rating Scale</td>
</tr>
<tr>
<td>VAMP7</td>
<td>Vesicle Associated Membrane Protein 7</td>
</tr>
<tr>
<td>Y1472</td>
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</tr>
<tr>
<td>Y1336</td>
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<tr>
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<td>Yeast artificial chromosome</td>
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<td>zQ175</td>
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Chapter 1: Introduction

1.1. Huntington Disease

Huntington disease (HD) is a fatal hereditary neurodegenerative disorder. It is caused by a CAG trinucleotide repeat expansion in the gene coding the protein huntingtin (HTT), resulting in a polyglutamine repeat expansion near the N-terminus of this protein (“A Novel Gene Containing a Trinucleotide Repeat That Is Expanded and Unstable on Huntington’s Disease Chromosomes. The Huntington's Disease Collaborative Research Group,” 1993). More than 35 repeats lead to HD, and the age of onset is inversely proportional to the number of CAG repeats, which encode an expanded polyglutamine (polyQ) in HTT (Langbehn et al., 2010). More than a decade before overt motor onset of HD, many gene mutation carriers exhibit impaired cognitive flexibility and deficits in skilled motor learning (Paulsen et al., 2008; Tabrizi et al., 2009). Furthermore, psychiatric disturbances such as depression, irritability, and apathy, as well as impaired executive function and other behavioural disturbances, are more common in both prodromal and manifest HD (Landwehrmeyer et al., 2016). In fact, suicide is among the leading cause of death in HD, and about a third of HD patients have a history of suicidal ideation (Honrath et al., 2018). GABAergic medium spiny neurons (MSN) of the striatum (specifically the D2 neurons, those involved in the indirect pathway that normally inhibit movement) are the first cell type of the brain to exhibit neurodegeneration (Mackay et al., 2018). This contributes to the typical chorea movements (involuntary hyperkinetic movement), followed later by a hypokinetic phase when the rest of the striatum degenerates. Dysregulation of synaptic function and calcium handling are common traits in HD (Mackay et al., 2018) as well as many other neurodegenerative disorders such as Alzheimer’s (Calderon-Garcidueñas & Duyckaerts, 2017; Liang et al., 2015; Small, 2009; Supnet
& Bezprozvanny, 2010) and Parkinson’s (Poewe et al., 2017; Verma et al., 2018; Zaichick et al., 2017).

1.1.1. Huntington Disease Clinical Presentation

Diagnosis of HD is dependent on genetic testing (associated with a family history), and the onset of motor symptoms such as chorea as defined by the Unified HD Rating Scale (UHDRS) total motor score (TMS). This score ranges from 0 (no motor symptoms) to 4 (motor symptoms most likely due to HD). The UHDRS-TMS includes many motor tests that assess eye movement, speech, controlled movements, chorea, and gait (Full test will be in the Appendix). The appearance of overt motor symptoms is also defined as manifest HD. More than a decade before motor symptoms, HD patients experience cognitive deficits and psychiatric symptoms. This time before clinical onset is called pre-manifest HD (Ross et al., 2014).

1.1.1.1. Motor symptoms

The progression of motor symptoms in HD follows a biphasic course which correlates with the preferential degeneration of the indirect pathway in the striatum followed by that of the direct pathway. In manifest HD, patients first exhibit a hyperkinetic phase characterised by chorea or involuntary dance-like movements. As the direct pathway of the striatum degenerates further, patients experience more of a hypokinetic/parkinsonian-like phenotype such as dystonia, bradykinesia, and gait disturbances (Rosenblatt et al., 2006).

1.1.1.2. Cognitive symptoms

HD patients experience cognitive symptoms even in premanifest HD. This decline is characterised by decreased processing speed, executive and visuospatial function, as well as
decreased ability to identify emotions based on facial features (Papoutsi et al., 2014). The most sensitive test for premanifest HD is the Stroop word reading which tests executive function and processing speed (Scarpina & Tagini, 2017).

1.1.1.3. Psychiatric symptoms

Many psychiatric conditions happen in manifest and also pre-manifest HD like anxiety, apathy, depression, obsessive compulsive disorder (OCD), and psychosis to varying degrees (Craufurd et al., 2001; Tabrizi et al., 2009). When looking at both manifest and pre-manifest HD patients, the REGISTRY study found that the most common psychiatric condition is apathy (28%), while other conditions like depression, irritability, and obsessive-compulsive disorder are present, but not as frequent (13% each). Psychosis is more rare, but still occurs in 1% of the cohort (van Duijn et al., 2014).

1.1.2. Huntingtin

The HTT gene encodes a well conserved 348-kDa protein. The polyQ stretch is at the N-terminal region. The polyQ stretch is preceded by 17 amino acids, and followed by a proline-rich domain (PRD). The N-terminal 17 amino acids are composed of an amphipathic alpha-helix. This structure is important in attaching to the endoplasmic reticulum and also functions as a nuclear export signal. This structure is prone to post-translational modifications that affect its clearance and subcellular localization. The PRD is found only in mammals, is variable even in the non-HD population, and is important for protein-protein interactions. In mice, deletion of the PRD in vivo has no effect on behaviour. The polyQ repeat is longer in mammals and in humans especially. In fact it is polymorphic in humans, as the repeat length varies from one human to the next. The effect of this variability in the number of Q repeats on HTT normal function is not well understood.
However, HTT function is known to be essential for life, as complete knock-out of huntingtin leads to prenatal death in mice (Dragatsis et al., 1998; Duyao et al., 1995; Saudou & Humbert, 2016).

1.1.2.1. Huntingtin Structure

The N-terminal region of HTT contains a 17Q stretch that can be in several conformations like an alpha-helix, random coil, and extended loop (Atwal et al., 2007; Kim et al., 2009). The PRD has a proline-proline helix that helps stabilise the polyQ structure. This helix may be important in the ability of mutant HTT to aggregate (Saudou & Humbert, 2016). The rest of the structure of HTT – 66 exons coding for amino acids 69 to 3144 or 97.8% of the protein – is not as well described. Exon 1 contains the well documented expanded polyQ stretch mutation, where most research is focused. The rest of the protein contains many HEAT repeats involved in protein-protein interactions. The name “HEAT” is an acronym for 4 proteins that contain this structure: HTT, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the serine/threonine-protein kinase TOR1. Between 16-36 HEAT repeats are grouped in 3-5 alpha-rod regions with disordered regions in between (Palidwor et al., 2009; Takano & Gusella, 2002). HEAT repeats are antiparallel alpha-helices with non-helical regions in between. These helices may adopt solenoid-like structures that can bind many protein complexes. The 507-1230 amino acid (AA) region of HTT can bind the N-terminal domain (1-506 AA) and C-terminal domain (2721-3144 AA) of other HTT proteins. The 507-1230 AA region can also bind one another (Palidwor et al., 2009). Furthermore, N-terminal regions of HTT can bind C-terminal regions of other HTT (1-416 with 1725-2800 and 1-586 with 2416-3144). Given this wide array of binding options between individual HTT proteins, HTT can adopt various 3D structures in up to 100 different conformations (Seong et al., 2010). Finally, the nuclear export signal (NES) motif at 2397-2406 regulates HTT localization (J. Xia et al., 2003).
1.1.2.2. *Huntingtin Function*

As previously stated, HTT function is essential for life (Dragatsis et al., 1998; Duyao et al., 1995), and many of these functions have started to be defined. To begin, HTT is an important player in the trafficking of vesicles. HTT interacts directly with dynein or indirectly through Huntingtin-associated protein 1 (HAP1) with the p150(Glued) subunit of dynactin and the kinesin family member 5C (KIF5C) (Caviston et al., 2007; Colin et al., 2008; Engelender, 1997; Gauthier et al., 2004; S. H. Li et al., 1998; McGuire et al., 2006; Strehlow et al., 2007; Twelvetrees et al., 2010). Through these interactions, HTT controls the transport of organelles in the anterograde and retrograde directions in axons and dendrites. These organelles include synaptic precursor vesicles, v-Soluble NSF Attachment Protein Receptor (SNARE) Vesicle Associated Membrane Protein 7 (VAMP7) protein containing vesicles, autophagosomes, endosomes, lysosomes, brain-derived neurotrophic factor (BDNF)-containing vesicles, amyloid precursor protein (APP)-positive vesicles, and gamma-aminobutyric acid (GABA)-receptor-containing vesicles (Colin et al., 2008; Gauthier et al., 2004; Liot et al., 2013; Wong & Holzbaur, 2014; Zala, Hinckelmann, & Saudou, 2013). HTT may enhance transport speed of vesicles along microtubules through binding of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on vesicles important for the energy provision of fast axonal transport (Zala, Hinckelmann, Yu, et al., 2013). HTT not only increases velocity of vesicle transport, but also affects the directionality of transport through phosphorylation of its S421 mediated by the kinases protein kinase B (Akt/PKB) and the serum- and glucocorticoid-induced kinase (SGK) (Colin et al., 2008; Humbert et al., 2002; Rangone et al., 2004). Calcineurin activation leads to its dephosphorylation (Pardo et al., 2006).

One key HTT function among several is its importance in the regulation of autophagy (Steffan, 2010). In HD, the abnormally long polyQ stretch enhances the autophagy pathway in
various HD models by inhibition of the mammalian target of rapamycin (mTOR), but loading of these autophagosomes is defective leading to the reduced ability of cells to effectively clear aggregated proteins and organelles (Kegel et al., 2000; Martin et al., 2014; Ravikumar et al., 2004). Interestingly, deletion of the polyQ stretch in mice enhances normal autophagy function and leads to increased longevity (Zheng et al., 2010).

Though HTT is mostly found in the cytoplasm, HTT can also be found in the nucleus. PolyQ tracts are found in transcription factors and function as transcription regulating domains. Given this information and some evidence in the literature, HTT plays an important role in the regulation of transcription of certain genes (Saudou & Humbert, 2016). HTT interacts with many transcription factors such as the cAMP-response element binding protein (CREB), NeuroD, the specificity protein-1 (SP1), the nuclear factor-kB (NF-kB), and the tumour-suppressor protein 53 (p53) (S.-H. Li et al., 2002; Marcora et al., 2003; Steffan et al., 2000; Takano & Gusella, 2002). Furthermore, HTT interacts with many transcriptional activators and repressors, nuclear receptors (LXRalpha, PPARgamma), vitamin D receptor, and thyroid hormone receptor-alpha 1 (TRalpha1) (Dunah et al., 2002; Futter et al., 2009; Holbert et al., 2001; Kegel et al., 2000; Yohrling et al., 2003). In fact, transcription dysregulation has been observed in post-mortem HD brains (Saudou & Humbert, 2016; Valor, 2015).

HTT also coordinates cell division as it is found in dividing cells at the spindle polls, mitotic spindles, and astral microtubules (Elias et al., 2014; Gutekunst et al., 1995). Here, HTT mediates spindle orientation (Godin et al., 2010). Silencing HTT in Drosophila leads to spindle misorientation in neuroblasts (Godin et al., 2010). HTT is also a player in regulating ciliogenesis, endocytosis, vesicle recycling, and endosomal trafficking (Haremaki et al., 2015; X. Li et al., 2008; Pal et al., 2006; Waelter et al., 2001).
1.1.3. HD Animal Models

Since the discovery that HD is caused by a single gene mutation – the presence of more than 35 CAG repeats in exon 1 of the HTT gene – the generation of powerful animal models became possible. But before this discovery, toxin models of HD existed and were studied. As such, HD models can be divided into non-genetic (toxin) and genetic models (Abada & Ellenbroek, 2016; Farshim & Bates, 2018).

1.1.3.1. Toxin Models

Toxin models of HD are among the earliest HD models in rats and mice. They were based on the finding that the post-mortem striatum of HD patients showed mitochondrial dysfunction and abnormalities in synaptic transmission. As such, acute or repeated exposure of excitatory agonists (e.g. kainic acid, quinolinic acid, 3- nitropropionic acid) became the HD model prior to genetic models. These toxin models more-or-less mimicked the striatal-selective degeneration, but were limited because they did not reproduce the production and accumulation of mutant HTT protein (Abada & Ellenbroek, 2016).

1.1.3.2. Genetic Models

These models have construct validity (i.e., because they include the mutant HTT gene) and generally recapitulate the pathological mechanisms and progressive nature of HD (face validity) more accurately than toxin models. They can be grouped into 3 models: 1) the fragment genetic mouse model of human HD, 2) the full-length human HD model, and 3) the knock-in HD mouse model (Abada & Ellenbroek, 2016; Farshim & Bates, 2018). The most relevant models will be discussed.
### 1.1.3.2.1. Fragment Genetic Mouse Model of Human HD

The first class of genetic mouse models of HD to come from the discovery of HD being caused by a single gene mutation is the fragment model. This model is defined by the expression of a single copy of a human genomic fragment that contains the HTT promoter sequences, exon 1 of HTT, and approximately 200 bp of intron 1. The R6 models of HD were among the first of this class (Mangiarini et al., 1996). Of this came the R6/1 and R6/2 model. Exon 1 of mutant HTT is expressed at 31% (R6/1) and 75% (R6/2) of the level of the endogenous protein. These translate to generate exon 1 of the mutant HTT protein (Mangiarini et al., 1996). The R6/1 mouse model had 116 CAG repeats whereas the R6/2 initially carried 150 CAG repeats. In fact, the number of CAG repeats in the R6/2 line varied depending on the background strain and whether transmission of the mutant allele occurs through the male or female (Mangiarini et al., 1997).

The R6/2 mouse line has an early disease onset and rapid disease progression compared to the other classes of genetic models. End-stage of the disease happens around 14-15 weeks of age, but varies depending on background mouse strain. Furthermore, HTT aggregates and inclusions appear in the R6/2 brain before any other phenotypes (Davies et al., 1997; Stack et al., 2005). R6/2 mice also develop motor and cognitive deficits by 5-6 weeks and 4 weeks post-natal respectively (Carter et al., 1999; Lione et al., 1999). Electrophysiological changes in the cortical-striatal pathway are also present at 4 weeks of age (Cepeda et al., 2003). At these ages, changes in transcription and changes in signalling pathways happen concurrently (Benn et al., 2008; Bibb et al., 2000; J. H. Cha et al., 1998). Skeletal muscle and cardiac pathology develop later (Mielcarek et al., 2014; Ribchester et al., 2004). Longer CAG typically correlates with earlier age of onset and more rapid disease progression. Mice with 110 CAG repeats intuitively have a more severe
phenotype compared to mice with 200 CAG repeats. Longer than 330 CAG repeats results in milder phenotype due to decreased mutant HTT levels and the lack of the aggregate pathology in the nucleus (Dragatsis et al., 2009).

Another mouse model of this class is the N171-82Q. This model has an N-terminally truncated human HTT protein encoded by a cDNA; it contains the first 171 amino acids with 82 CAGs regulated by a mouse prion promoter. These mice have no behavioural deficit at birth, but they develop tremors, hypokinesia, and lack of coordination. They fail to gain weight, and live for about 6 months (Farshim & Bates, 2018; Ribchester et al., 2004).

1.1.3.2. Full-length Human HD Model

This class of genetic mouse models was generated by introducing mutant HTT in the form of yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC). These models develop disease phenotypes more gradually than fragment genetic mouse models, and show similar lifespans to wild-type mice (Abada & Ellenbroek, 2016; Farshim & Bates, 2018). The YAC128 model expresses 4 copies of human HTT with 125 CAG repeats that are interrupted by CAACACACACAGCAA at positions 24-28 and 109-113 (Pouladi et al., 2012). It is available on both FVB/N and C57BL/6 strain backgrounds. Between these two strains, the FVB/N line has the more severe phenotype (Van Raamsdonk et al., 2007). Motor and cognitive deficits, neuronal loss and striatal atrophy develop after 3 months (Slow et al., 2003). The YAC128 model also suffers motor learning deficits as evidenced by a slower learning of the rotarod task as early as 4 weeks of age on this task (Brooks et al., 2012; L. Menalled et al., 2009). Aggregate pathology and neuronal inclusions also occur in the brain (Bayram-Weston et al., 2012).
Briefly, the BACHD mouse model shares many similarities with the YAC128 model. Like the YAC models, BACHD mice display a progressive phenotype showing behavioural deficits at 2 to 6 months of age, but more pronounced at 12 months. They are also very similar in electrophysiological deficits in cortical pyramidal neurons, cortical interneurons, and medium spiny neurons. Synaptic pathology occurs early and progressively worsens at 6 months (Gray et al., 2008).

HD patients normally experience weight loss, but full-length models experience weight gain, which might be mediated by insulin-like growth factor 1 expression (because of full-length dosage) (Pouladi et al., 2010). Given this, The YAC128 model cannot be used to assess metabolic changes in HD. Moreover, unlike the R6 line, YAC128 mouse line is resistant to germ-line and somatic instability (probably due to the presence of nine CAA codons within the CAG repeat) (Farshim & Bates, 2018; Gu et al., 2022). Though this means that one aspect of HD is missing as somatic instability is present in the HD patient population, this same trait also makes it much easier to maintain YAC128 colonies with a stable phenotype. Furthermore, this stability leads to results gathered from the YAC128 mouse line to be more reproducible and less variable as the CAG repeat length can have strong effects on the rate of progression of HD (Dragatsis et al., 2009). Also, given the slower progression of HD in YAC128 versus the R6 line, it makes the YAC line a more appropriate model to study cellular mechanisms in presymptomatic HD (Slow et al., 2003).

1.1.3.2.3. Knock-In (KI) Mouse Model

These models consist of allelic lines (e.g. HdHQ150 or zQ175 lines) that enable probing the role of polyQ length as distinct from other confounders of different lines (transgenics, background strain, etc). These lines have improved construct validity as they don't overexpress
HTT (unlike the transgenic lines). Though there are various KI models, I will be summarising them in Table 1 and briefly discussing the HdhQ150 and zQ175 KI lines.

Briefly, the HdhQ150 mouse line (aka CHL2) has 150 CAG repeats that replaces the polyQ sequence of (CAG)$_2$CAA(CAG)$_4$ of the murine allele. Only the CAG tract was different between the targeted and normal allele (Lin et al., 2001). These can be used as heterozygotes or homozygotes. C57BL/6 and CBA lines have been developed (Woodman et al., 2007). Like the fragment genetic mouse models, the CAG repeat length is unstable on gametic transmission, especially on C57BL/6 background. Many versions of this model with varying CAG lengths have been generated: 50, 100, 150, 200, 250, 315, and 365 (Farshim & Bates, 2018; L. Menalled & Chesselet, 2014).

The zQ175 KI line is the only model where robust phenotypes are observed in heterozygous mice compared to other KI models (Farshim & Bates, 2018). Briefly, homozygous mice display abnormalities earlier than heterozygous mice. Homozygous mice begin to show gait abnormalities at 4 weeks of age and motor deficits at 8 weeks of age. Rotarod and climbing abnormalities occur at 30 weeks and cognitive deficits at 12 months. As expected, heterozygous mice display motor deficits from around 4-5 months of age, which is much later than in the case of homozygous mice (L. B. Menalled et al., 2012).

1.2. Clinical Trials

As described previously, HD is characterised by an initial involuntary hyperkinetic movement followed by a hypokinetic phase. Currently, HD is clinically diagnosed at the onset of these involuntary chorea movements, and the only FDA approved treatments for these patients are
tetrabenazine and deutetetrabenazine, which are used to treat these uncontrollable movements. Tetrabenazine acts by decreasing dopamine at the synapse by inhibiting vesicular monoamine transporter type 2. This leads to a decrease in input from the substantia nigra pars compacta to the striatum, and an attenuation of uncontrollable movement. Tetrabenazine serves as a symptomatic treatment for chorea, but does not display any disease-modifying effects. Once patients reach the hypokinetic phase of the disease, tetrabenazine is no longer useful. Unfortunately, disease-modifying treatments are not currently available for HD (Potkin & Potkin, 2018) Below, a summary of various clinical trials regarding the treatment of HD will be discussed to portray a picture of where pharmacological treatment stands at the moment.

1.2.1. Memantine Trials

Clinical testing of memantine was on the findings that blocking NMDA receptors could alleviate motor symptoms and chorea induced by levodopa in parkinsonian animal models (Blanchet et al., 1998, 1999; Papa et al., 1995), and the involvement of glutamate toxicity in HD and other neurodegenerative disorders. Amantadine, an NMDA receptor antagonist, was then used in a clinical trial to test its effect on motor and cognitive function in HD (Verhagen Metman et al., 2002). The results showed a reduction in chorea, but no change in cognitive measures.

These findings eventually lead to the use of memantine – which has a similar effect as amantadine – in a clinical trial. Memantine is a noncompetitive NMDA receptor antagonist. It was considered due to its ability to stabilise NMDA receptor tone. It was eventually discovered after the clinical trial that memantine preferentially blocks extrasynaptic NMDA receptors (P. Xia et al., 2010) which was shown to be increased early in YAC128 mice (Milnerwood et al., 2010). Briefly, the main eligibility criteria was that participants were able to ambulate and also exhibited
chorea – these can also be described as patients that are in the relatively early phases of manifest HD. 12 patients underwent a trial of 5 to 20 mg/day memantine in 2 doses over 4 weeks, then followed for 3 months. Other medications were unchanged during the trial. Change in the UHDRS score was used as the primary outcome. 3 patients stopped midway through the trial because of either no obvious improvement detected, no follow-up, or combined lack of efficacy and adverse events (confusion/hallucinations). The rest of the patients completed the trial. Like the amantadine trial, there were improvements in motor score as evidenced by the detected difference in UHDRS motor score (p = 0.008). This difference was mostly due to decrease in maximum chorea rating (p = 0.008). There was however no difference in cognition (p = 0.625), behavior (p = 0.258), total functional capacity (p = 0.078), and independence scale rating (p = 1.000). No serious adverse events were reported. As of now, memantine is not approved for use in HD (Ondo et al., 2007).

1.2.2. Pridopidine Trials

Recently, there have been clinical trials for pridopidine, a S1R agonist. Briefly, the S1R is a 28 kDa transmembrane protein located in the endoplasmic reticulum (ER) membrane, but specifically enriched at regions contacting mitochondria. It is also found in nuclear and plasma membranes (Chu & Ruoho, 2016; Mavlyutov et al., 2015; Su et al., 2010; Tsai et al., 2015). Activation of S1Rs by ligand binding is involved in stabilizing calcium homeostasis at the ER-mitochondrial membrane (Su et al., 2010; Tchedre et al., 2008; K. Zhang et al., 2017).

Phase II trials of pridopidine suggest it ameliorates motor deficits to a small degree in diagnosed HD, but in post-hoc analyses, it was shown to significantly slow decline in functional capacity (MermaiHD, HART, Pride-HD, Teva) (de Yebenes et al., 2011; Huntington Study Group HART Investigators, 2013; Reilmann et al., 2019). Briefly, the MermaiHD trial was a phase 3
study in Europe that compared 2 doses of pridopidine (45 mg and 90 mg) over the course of 6 months in 437 patients with manifest HD. The 90 mg dose showed improvements in motor score according to the UHDRS scale, and pridopidine was well tolerated. There were however no changes in other measures like cognition (de Yebenes et al., 2011).

The HART study was a similar study done in North America. 3 doses of pridopidine were compared (20 mg, 45 mg, and 90 mg) in 227 patients with manifest HD over the course of 3 months. Once again, pridopidine was well tolerated, but only the 90 mg showed improvements in motor score, and no significant changes in other outcome measures such as cognitive, behavioural, functional, or global measures were observed (Huntington Study Group HART Investigators, 2013).

Finally, the PRIDE-HD trial was a phase 2 clinical trial that tested a higher dose of pridopidine. 4 doses were tested: 45 mg, 67.5 mg, 90 mg, and 112.5 mg. This time, patients with manifest HD were being treated for 52 weeks, though the primary outcome measure was a change in the motor score on the UHDRS scale after 26 weeks of treatment. Unfortunately, results from previous studies could not be replicated, as there were no changes between treatment and placebo (Reilmann et al., 2019). However, there was a significant change observed in total functional capacity with the 90 mg pridopidine dose after 52 week of treatment (McGarry et al., 2020).

There is currently a Phase 3 trial underway of pridopidine in manifest HD (early to mid) (NCT04556656). The patient inclusion criteria includes the presence of enough motor signs in order to be able to detect improvements. This study will be the longest, lasting 65 weeks. Furthermore, another clinical trial testing the safety and efficacy of pridopidine treatment of ALS is also underway.
Overall, these results show minimal improvements and patients were only followed for at most one year. Furthermore, these patients were treated once clinically diagnosed, whereas prior to motor symptoms, HD patients exhibit cognitive deficits for at least a decade. Thus, the disease would have already progressed significantly by clinical diagnosis. Given that pridopidine has shown neuroprotective properties in pre-manifest mouse models of HD, the idea that it is a disease-modifying drug has emerged. Furthermore, given the autosomal dominant nature of HD, early intervention with genetic testing is feasible.

1.2.3. Antisense Oligonucleotide Trials

Given the genetic nature of HD, targeting the expression of mutant HTT directly has been another front to tackle this disease. One way of decreasing its expression is through the use of antisense oligonucleotides (ASOs). The IONIS-HTT<sub>Rx</sub> is one such ASO developed to target the HTT mRNA. Upon binding their target through Watson-Crick base pairing, this hybridization results in endogenous RNase H1-mediated degradation which inhibits the translation of both the HTT and mutant HTT.

The IONIS-HTT<sub>Rx</sub> was tested in two clinical trials. The first trial (4 months) showed proof of target engagement (dose-dependent mutant HTT lowering in the CSF) and tolerability (Tabrizi et al., 2019). The second trial was Phase 3 and tested the highest ASO dose at two different dosing intervals vs placebo; that trial was halted because it showed faster progression in the more frequent dosing group, but data analysis for secondary outcome measures is ongoing.

1.2.4. CRISPR-Cas9

Beyond ASOs, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9) has emerged as an enticing therapeutic approach to directly modify
the mutant HTT gene altogether. There are currently no trials underway, but it was shown that the CRISPR-Cas9 system packaged with a single guide RNA into a single adeno-associated virus (AAV) vector could disrupt the mutant HTT gene and cause an increased lifespan and improved motor performance in R6/2 mice (Ekman et al., 2019).

Though HD can be genetically targeted, understanding its cellular mechanistic underpinnings and pharmacological treatments is still very useful. They could be used as adjunct therapy, and studying neurodegeneration with the HD mouse models offers a powerful tool to also understand other neurodegenerative disorders like Alzheimer’s and Parkinson’s as they share many mechanistic underpinnings such as altered calcium homeostasis and excitotoxicity.

1.3. Calcium Handling

One feature of mouse models of HD is the dysregulation of calcium at the level of the ER (Mackay et al., 2018), whereby mutant HTT directly interacts with and sensitises the type 1 inositol (1,4,5)-trisphosphate receptor (IP3R1, a calcium channel at the ER membrane) to IP3 (I. Bezprozvanny, 2011). The IP3R is a calcium channel located on the ER membrane that plays an important role in neuronal calcium signalling. Of the three isoforms, IP3R1 is the most common type found in neurons (Egorova & Bezprozvanny, 2018). The sensitization of IP3R1 to IP3 leads to excessive calcium release from the ER and thus calcium store depletion. This triggers the store-operated calcium response (SOCR) which is the refilling of ER calcium stores using plasma membrane calcium channels (I. Bezprozvanny, 2011). Briefly, STIM (stromal interaction molecule), a single pass transmembrane protein located on the ER membrane, detects low calcium in the ER with an EF hand domain and recruits store-operated calcium channels located on the
plasma membrane. Excessive ER calcium release mediated by IP3R1 thus causes excessive calcium store refilling (Geva et al., 2016; Vigont et al., 2015) leading to altered calcium dynamics. Interestingly, activating sigma-1 receptors (S1R) – transmembrane proteins located in the ER membrane and well-poised to modulate ER calcium handling – has been shown to normalise these aberrant features of ER calcium dynamics in HD (Ryskamp et al., 2017).

1.3.1. Endoplasmic Reticulum (ER)

The ER is a special cellular organelle involved with protein, lipid and steroid synthesis, carbohydrate metabolism, and calcium homeostasis (English & Voeltz, 2013). Given this vast list of functions, the ER interacts with many other organelles, including the plasma membrane, the Golgi apparatus, peroxisomes, and the mitochondria (Schwarz & Blower, 2016). This implies the ER’s massive size, and this is indeed the case in neurons; in fact, the ER extends from the soma to the entire dendritic tree, to most synaptic spines, and throughout the axon (Y. Wu et al., 2017).

The ER contains the cell’s main calcium stores, and is in close proximity with the plasma membrane for various tasks involving calcium homeostasis (Ghemrawi & Khair, 2020). This is the one of the reasons behind the tight coupling of calcium-induced calcium release and the SOCR. The SOCR is the refilling of calcium stores when ER calcium is depleted. This depletion could happen via the opening of IP3 receptors or ryanodine receptors (RYR) (Ghemrawi & Khair, 2020; Kikuta et al., 2019; Schwarz & Blower, 2016).

1.3.1.1. IP3 Receptors

In non-excitable cells, IP3 receptor generated calcium signals are important for regulating metabolism, secretion, fertilisation, proliferation, and smooth muscle contractions (Berridge, 2016; Mikoshiba, 2015). In excitable cells, calcium signals depend mainly on voltage-gated
calcium channels, calcium release through ryanodine receptors via calcium-induced calcium release, and NMDA receptors in neurons – important for muscle cell contraction, memory formation, insulin secretion from beta cells (Berridge, 2016; Mackay et al., 2018). Interestingly, in excitable cells, IP3 receptors seem to have a lesser role and may be involved in subtly modifying the main calcium signal (Berridge, 2016).

IP3 is produced via activation of G protein-coupled receptors (GPCRs) (e.g. type 1/5 metabotropic glutamate receptor (mGluR1/5)) or protein tyrosine kinase-linked receptors (PTKRs) coupled to phospholipase C (PLC) (Cocco et al., 2015). Activated PLC hydrolyzes lipid phosphatidylinositol 4,5-bisphosphate to both diacylglycerol (DAG) and IP3. IP3 then binds IP3 receptors (located on the endoplasmic reticulum membrane) and sensitises them to cytosolic calcium and leads to calcium flux from the ER lumen to the cytosol. IP3 is degraded by an IP3 3-kinase or inositol polyphosphate 5-phosphatase (Connolly et al., 1987). The inositol generated by this metabolism is recycled to make more phosphatidylinositol.

IP3 receptors are tetramers that form an ion channel embedded in the ER and the nuclear envelope. There are 3 isoforms: IP3R1, IP3R2, and IP3R3. They share very similar structures but have different gating properties leading to different temporal and spatial characteristics of the ensuing calcium signal (Taylor et al., 2004, 2014). In order for IP3 receptors to gate, IP3 has to bind each of the four subunits (Alzayady et al., 2016). A conformational change is then induced and which reveals a calcium binding site. The channel opens when calcium binds to all 4 sites. At high cytosolic calcium concentrations (300 nM), calcium binds to another site on the IP3 receptor subunits which causes them to close. Because of this, IP3 receptors typically are only open very briefly, and the observed calcium transients are also very brief (Berridge, 2016; Taylor et al., 2004).
In HD, mutant HTT directly binds to IP3 receptor cytosolically and sensitises it to IP3 (Tang et al., 2003). This may lead to calcium transients from IP3 receptors being more frequent and potentially more sustained. Primary rat striatal MSN cultures transfected with mutant HTT show increased calcium events in response to a non-saturating dose of the mGluR1/5 agonist 3,5-dihydroxyphenylglycine (DHPG) (Ryskamp et al., 2017; Tang et al., 2003). Mutant HTT directly binding IP3 receptors implies that enhanced IP3 receptor function happens at an early stage of the disease time course before any neurodegeneration occurs. Interestingly, some evidence has emerged that normalising IP3 receptor function ameliorates neurodegeneration. YAC128 MSN cultures show enhanced calcium signals in response to repetitive glutamate application – increased calcium response is associated with apoptosis – and this response is prevented by direct IP3R or mGluR blockade (Tang et al., 2005). Moreover, IP3 receptor KD with an antisense RNA prevented synapse loss in YAC128 cortical-striatal co-cultures (Mackay et al., 2018; J. Wu et al., 2016).

1.3.1.2. Store-Operated Calcium Response (SOCR)

Overactive leak from IP3 receptors also leads to elevated SOCR, a compensatory mechanism that refills the ER with calcium (J. Wu et al., 2016). In YAC128 MSNs, the SOCR is mediated by the stromal interacting protein type-1 and 2 (STIM1 and STIM2), calcium sensors embedded in the ER membrane sensing calcium in the ER lumen with its EF hand motif (Kikuta et al., 2019; Mackay et al., 2018; J. Wu et al., 2016). Lower ER calcium levels leads to the recruitment of sarco-endoplasmic reticulum calcium ATPase pumps (SERCA) – a calcium pump located on the ER membrane – and store-operated calcium (SOC) channels – calcium channels located on the plasma membrane in close proximity to the SERCA pumps (Mackay et al., 2018; Prakriya & Lewis, 2015). Only 10 nanometers separate the plasma and ER membrane, positioning these SOC channels in close enough proximity such that the ER can be refilled with calcium from
the extracellular space with only a very transient and local increase in cytosolic calcium (Prakriya & Lewis, 2015).

Pharmacological SOCR blockade prevents MSN spine loss, but does not prevent glutamate-mediated apoptosis (Czeredys et al., 2017; J. Wu et al., 2016). SOC may hence play a role in neurodegeneration. Altogether, IP3 receptors are aberrantly overactive in HD, and normalising its function and any consequence of that increased function is definitely an avenue for therapeutic intervention.

1.3.1.3. Ryanodine Receptors

Ryanodine receptors (RyR) are homologous tetrameric calcium channels found on the endoplasmic/sarcoplasmic reticulum ubiquitously expressed in all cell types (Hertle & Yeckel, 2007; H. H. Wu et al., 2011). There are three isoforms – RyR1, RyR2. RyR3. RyR1 are mostly found in skeletal muscle, RyR2 are found in cardiac muscle and the main neuronal subtype, and RyR3 were originally identified in the brain, but are also widely expressed in different cell types (Abu-Omar et al., 2018; Santulli et al., 2018). They are the biggest ion channel in the body, with each subunit being ~500 kDa making the whole protein more than 2000 kDa (Santulli et al., 2018).

Briefly, in muscle cells the gating of RyR1 is dependent on a mechanical coupling with the opening of an L-type voltage gated calcium channel (CaV), CaV1.1. This amplifies the calcium from CaV channels by translating their opening to mechanical energy to open RyR1 channels, leading to a flux of calcium from the sarcoplasmic reticulum (Nelson et al., 2013; Rios & Brum, 1987; Santulli et al., 2018; Santulli & Marks, 2015). This marks a subtle but important difference between RyR1 and RyR2, which predominates in neurons. RyR2 are not directly coupled to CaV channels; instead, the calcium itself entering the cell via CaV opening is the requirement for gating
RyR2 (Abu-Omar et al., 2018; Santulli et al., 2018). Each subunit of RyR2 has a calcium binding site facing the cytosol that when bound, leads to calcium-induced calcium release (CICR) – the opening of RyR due to an increase in cytosolic calcium. For each subunit, there are actually 2 calcium binding domains: a high and low affinity site. Calcium-binding to the high affinity site gates RyR2 via CICR, and once the cytosolic calcium concentration reaches ~100 uM, calcium binds the low affinity site leading to closure of RyR2 (L. Bezprozvanny et al., 1991; Santulli et al., 2018). Due to this gating mechanism, calcium signals from RyR are transient, having an open time between 1-10 ms (Fill & Gillespie, 2018). The closed state of RyR is stabilised by the calcium channel-binding and stabilising proteins calstabin1 and calstabin2, also known as FK506-binding protein 12 and FK506-binding protein 12.6 respectively (FKBP12 and FKBP12.6) (Brillantes et al., 1994; Santulli et al., 2018).

Like IP3 receptors, RyRs are also leaky in HD. In fact, blockade of RyRs in rat cortical and striatal cultures using dantrolene was able to reduce cell death from transfection with EGFP-tagged truncated N-terminal huntingtin 150Q. Overexpression of mutant HTT in HEK293t cells also led to an increased calcium leak after treatment with thapsigargin (SERCA inhibitor), and 2-Aminoethoxydiphenyl borate (2APB) (IP3 receptor inhibitor). Furthermore, abnormal calcium leak was also observed in cortical and striatal dissociated neurons from 14 week-old R6/2 mice. Finally, overexpression of the RyR stabiliser FKBP12 eliminated the calcium leak observed in the HEK293t cells, and decreased the proportion of cell death observed in the rat cortical and striatal cultures (Suzuki et al., 2012). Interestingly, FKBP12 mRNA levels were reported to be decreased in the caudate nucleus of grade 1 HD patients (before gross striatal atrophy) (Hodges et al., 2006).
1.4. Synapse-to-Nucleus Calcium Signalling

Synapse-to-nucleus signalling is a very important topic in neuroscience as it is the basis of many mechanisms of synaptic development and plasticity (Alberini, 2009; Greer & Greenberg, 2008). There are multiple ways by which a synaptic signal reaches the nucleus. The first way is through protein messengers via passive or facilitated diffusion through the nuclear pore, or via active transport (signalling endosomes or active retrograde transport of messenger protein), and the second way is via direct calcium signalling to the nucleus (Karpova et al., 2012). Briefly, protein messengers include the mitogen-activated protein kinases-extracellular signal-regulated kinase (MAPK-ERK) pathway, whereby ERK enters the nucleus via facilitated diffusion, and CREB which has been shown to be associated with endosomes.

Calcium signalling to the nucleus can happen through various mechanisms. For example, synaptic activity-induced action potentials can cause L-type CaV opening at the soma which would lead to a calcium influx that would cross the nuclear membrane (Bading, 2013). In this way, calcium is a reporter of the electrical activity of the neuron. One other method by which the synapse can communicate with the nucleus is through regenerative ER calcium waves (Bading, 2013; Hagenston & Bading, 2011; Karpova et al., 2012) – a mechanism by which CICR from the ER membrane leads to neighbouring ryanodine receptors on the ER membrane to undergo CICR all the way to the nucleus. In HD, due to the calcium leak through both IP3 receptors and RyR, it is likely that synapse-to-nuclear calcium signalling is altered given the aberrant calcium handling at the ER previously discussed (Mackay et al., 2018). Calcium is an important intracellular messenger in neurons, linking synaptic activity and cell excitability to gene expression (Bading et al., 1993; Bengtson & Bading, 2012; Hardingham et al., 1997; Lau et al., 2015; Wayman et al., 2008; S.-J. Zhang et al., 2007); indeed, transcriptional dysregulation is a hallmark of HD and
exploring alterations in synapse-to-nuclear calcium signalling may reveal a therapeutic avenue for this terrible disease (J.-H. J. Cha, 2007; Gallardo-Orihuela et al., 2019; Kumar et al., 2014; Malla et al., 2021; Steffan et al., 2000). See Figure 1.1 for a schematic of the different synapse-to-nucleus signalling mechanisms.

**Figure 1.1: Cartoon diagram depicting different synapse-to-nucleus signalling mechanisms.**
1.5. NMDA Receptors

Glutamate is the main excitatory neurotransmitter in the CNS. There are 3 main ionotropic glutamate receptors: the α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA) receptors, kainate receptors, and N-methyl D-aspartate (NMDA) receptors, named after the specific agonist. Furthermore, each class of glutamate receptors have their own group of subunits. NMDA receptors have several features that distinguish them from other glutamate receptors. They can act as coincidence detectors, since opening of this channel requires the voltage-dependent unblocking of extracellular magnesium, as well as the binding of both glutamate and glycine/D-serine. Furthermore, NMDA receptors are highly permeable to calcium, implying their importance in many cellular functions. NMDA receptor activation leads to an inward current thus depolarizing the membrane with a reversal potential of around 0 mV (Traynelis et al., 2010).

1.5.1. Synaptic versus Extrasynaptic NMDA Receptors

NMDA receptors are cationic channels permeable to sodium, potassium, and calcium, and are important in excitatory neurotransmission regulation in the central nervous system, as well as various plasticity mechanisms. They are heterotetramers formed from two GluN1 subunits and either two GluN2 subunits (GluN2A – GluN2D) or a GluN2 and GluN3 subunit (GluN3A – GluN3B) (Traynelis et al., 2010). Genetic deletion of the GluN1 subunit has been shown to cause death in neonate mice (Forrest et al., 1994), whereas excessive NMDA receptor activation has been shown to lead to excitotoxicity, aberrant calcium signalling, and eventually cell death (Arundine & Tymianski, 2004; Epstein et al., 1994; M. M. Y. Fan & Raymond, 2007). The initial model to describe these observations was that too little or too much NMDA receptor activation was potentially harmful to the neuron (Hardingham & Bading, 2010; Lipton & Kater, 1989). A
newer proposed model describes opposing roles of synaptic versus extrasynaptic NMDA receptors. On one hand, synaptic NMDA receptor activation stimulates neuroprotective signalling pathways required for synaptogenesis, synaptic plasticity involved in learning and memory, and activation of pro-survival gene transcription that maintain neuronal health. On the other hand, extrasynaptic NMDA receptor activation – due to excess glutamate which also activates NMDA receptors at extrasynaptic sites – opposes plasticity and promotes cell death pathways (Hardingham et al., 2002; Hardingham & Bading, 2010). For example, calcium entry through synaptic NMDA receptors leads to the activation of calcium/calmodulin-dependent protein (CaM) kinase IV which then phosphorylates and activates the transcription factor cyclic-AMP response element binding protein (CREB) at site Ser133 (Hardingham et al., 2001; Hardingham & Bading, 2010; Sun et al., 1994). This pathway leads to the transcription of prosurvival genes such as brain-derived neurotrophic factor (BDNF) which has neuroprotective properties (Hansen et al., 2004; Thoenen et al., 1987). On the contrary, extrasynaptic NMDA receptor activation leads to CREB dephosphorylation via the juxtasyaptic attractor of caldendrin on dendritic boutons protein (Jacob) (Hardingham & Bading, 2002). See Figure 1.2 for a schematic comparing synaptic vs extrasynaptic NMDA receptor activation. Interestingly, 1 week old rodents begin expressing GluN2A-containing NMDA receptors predominantly at synaptic sites. By adulthood, GluN2A-containing NMDA receptors become enriched at the synapse whereas GluN2B-containing NMDA receptors are more abundant at extrasynaptic sites. It has been proposed that GluN2A and GluN2B-containing NMDA receptors promote cell survival and cell death respectively (Liu et al., 2007), mimicking the data relating to their membrane localisation. However, other data show that either NMDA receptor subtype can both activate pathways resulting in cell survival or death (Martel et
al., 2009; von Engelhardt et al., 2007). The parameters that determine if GluN2 subunits promote cell survival or death still need to be elucidated.

Figure 1.2: Cartoon diagram depicting the difference between synaptic and extrasynaptic NMDA receptor activation. Reprinted from Neuron, 82(2), Parsons, M. P., & Raymond, L. A., Extrasynaptic NMDA receptor involvement in central nervous system disorders, 279–293, Copyright (2014), with permission from Elsevier.

1.5.2. NMDA Receptor Trafficking

Many factors determine the localisation of NMDA receptors. For example, the C-terminus of NMDA receptors is critical for protein-protein interaction, including with postsynaptic density protein 95 (PSD95), a scaffolding protein at the synapse. Cleavage of the C-terminus by the calcium-activated protease, calpain, excludes NMDA receptors from synaptic targeting (Doshi & Lynch, 2009; Gladding & Raymond, 2011; H. Mori et al., 1998; Steigerwald et al., 2000). One
more mechanism affecting localisation of NMDA receptors is palmitoylation of PSD-95 – required for synaptic targeting, and depalmitoylation of PSD-95 may reduce its ability to cluster at synapses and anchor NMDA receptors (Christopherson et al., 2003; Gladding & Raymond, 2011).

Another mechanism of NMDA receptor trafficking is tyrosine phosphorylation of NMDA receptors. Phosphorylation of the tyrosine 1472 (Y1472) residue of the GluN2B subunit by the tyrosine kinase Fyn prevents binding to the adaptor protein AP-2, leading to synaptic localisation (Prybylowski et al., 2005). Furthermore, PSD-95 also binds to the protein tyrosine kinase fyn, keeping it in close proximity to NMDA receptors (Ali & Salter, 2001; Tezuka et al., 1999). The striatal-enriched protein tyrosine phosphatase (STEP) can dephosphorylate the GluN2B Y1472 residue, leading to increased binding of AP-2, resulting in the translocation of GluN2B-containing NMDA receptors from synaptic to extrasynaptic sites (Braithwaite et al., 2006; Pelkey et al., 2002; Snyder et al., 2005). Furthermore, phosphorylation of the GluN2B Y1336 residue also leads to extrasynaptic localisation of GluN2B-containing NMDA receptors (Goebel-Goody et al., 2009). Serine and threonine residues are also sites for phosphorylation. GluN2B serine 1480 (S1480) phosphorylation by casein kinase II (CK2) decreases NMDA receptor interactions with PSD-95, facilitating GluN2B Y1472 dephosphorylation (Chung et al., 2004). This leads to GluN2B-containing NMDA receptors to no longer be anchored at the synapse (Gladding & Raymond, 2011; Sanz-Clemente et al., 2010).

Increased STEP activity is present in YAC128 HD mice, leading to reduced GluN2B Y1472 phosphorylation (Gladding et al., 2012). This contributes to an increase in extrasynaptic GluN2B-containing NMDA receptor numbers at HD cortical-striatal synapses, which also alters calcium signalling (Mackay et al., 2018; Milnerwood et al., 2012; Milnerwood & Raymond, 2010; Raymond, 2017). However, it is unclear how mutant HTT mediates this increase in extrasynaptic
NMDA receptors. One lab has shown that the transcriptional coactivator PGC-1α is underexpressed in HD, and that exogenous expression of PGC-1α leads to a decrease in extrasynaptic NMDA receptor currents without affecting synaptic NMDA receptors. Furthermore, the effects of mutant HTT and PGC-1α knockdown on extrasynaptic NMDA receptor activity occluded each other (Puddifoot et al., 2012). This presents a link between mutant HTT and extrasynaptic NMDA receptors, but the mechanism remains to be fully elucidated.

1.6. Activin A

Activin A is a dimer of inhibin-β-A subunits that is secreted and binds to plasma membrane receptors belonging to a serine/threonine kinase family known as type I (low molecular weight) and type II (high molecular weight) activin receptors (ACVRs) (Lau et al., 2015). It is expressed ubiquitously and has effects in other systems beyond the nervous system. Most research on Activin A has been done in reproductive physiology, whereby it is involved in the human foetal ovary development as well as the stages of follicular and luteal development (Bloise et al., 2019). Regarding the brain, Activin A has been demonstrated to be neuroprotective. Activin A is able to prevent apoptosis through inhibition of caspase-3 (Kupershmidt et al., 2007). Furthermore, Activin A has been shown to be an important neurotrophic factor, regulating dendritic spine morphology and modulating glutamatergic neurotransmission (Müller et al., 2006). Activin A also has antioxidant properties, is anti-inflammatory, and can promote neurogenesis (Abdipranoto-Cowley et al., 2009).

Recent work has shown that Activin A, a member of the transforming growth factor β (TGF-β) family whose transcription is nuclear-calcium-dependent, reduces toxic extrasynaptic
NMDA receptor signalling in the hippocampus in a mouse stroke model (Lau et al., 2015). Specifically, the Bading lab has shown that synaptic NMDA receptor activation leads to nuclear calcium signalling which then promotes the transcription of Activin A, which is secreted and binds ALK4 (activin-like kinase 4, also known as Activin receptor 1B [ACVR1B]). This leads to the reduction of toxic extrasynaptic NMDA receptor calcium influx as evidenced by Fluo-3 calcium imaging experiments using dizocilpine (MK-801) to block synaptic NMDA receptors, and in the presence of blockers of voltage-gated calcium channels and action potentials (Lau et al., 2015). Furthermore, neurons treated with Activin A led to a decreased phosphorylation of a tyrosine residue 1472 on the GluN2B subunit (Lau et al., 2015). Dephosphorylation at this site is known to induce endocytosis of GluN2B-containing NMDA receptors to reduce surface expression (T. Nakazawa et al., 2001). These studies however did not examine NMDA receptor trafficking directly, and the mechanism by which this dephosphorylation happens is not well understood considering that the activin A receptor functions as a serine/threonine kinase. Nevertheless, activin A’s neuroprotective effect on NMDA receptor calcium signalling cannot be understated and is an exciting therapeutic avenue for HD and other neurodegenerative diseases.

1.7. Sigma-1 Receptors

The S1R is a 28 kDa transmembrane protein located at the ER membrane, but also at nuclear, mitochondrial, and plasma membranes. They are also ubiquitously expressed throughout the CNS and in many cell types (Matsumoto et al., 2007). In response to ligand binding, the S1R can localise to different organelles, and they modulate a wide variety of ion channels and other proteins such as IP3 receptors, protein kinases, other calcium channels, voltage-gated sodium and
potassium channels, and NMDA receptors (Kourrich et al., 2012; Nguyen et al., 2015; Su et al., 2010). The S1R has such a varied role in modulating ion channels that it is very difficult to predict how these combined effects would impact overall neuronal excitability. The S1R is also involved in regulating ER stress responses derived from an accumulation of unfolded proteins (Hayashi, 2019; K. Mori, 2015; Su et al., 2010). Though the S1R has many known functions, how they interact together and affect the cell as a whole remains to be fully understood.

Though the S1R endogenous ligand is still unknown, many agonists and antagonists for research use are available. Strictly speaking, these compounds have been shown to bind to S1Rs and their classification of agonists/antagonists relies mostly on their ability to recapitulate effects of overexpressing (for agonists) or knocking down (for antagonists) S1Rs. Commonly used agonists in research are (+)-pentazocine, (+)-SKF10,047, PRE084 (2-morpholin-4-ylethyl 1-phenylcyclohexane-1-carboxylate), 3-PPP (N-n-propyl-3-(3-hydroxyphenyl) piperidine) and SA4503 (1-[2-(3,4-dimethoxyphenyl) ethyl]-4- (3-phenylpropyl)piperazine). Commonly used antagonists in research are BD1047 (N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine), BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine), and NE-100 (4-methoxy-3-(2-phenyl- ethoxy)-N,N-dipropylbenzeneethanamine). Furthermore, S1Rs have been shown to exhibit neuroprotective and calcium normalisation effects in HD as well as other neurodegenerative diseases such as Alzheimer’s disease (Maurice & Goguadze, 2017) and Parkinson’s disease (Francardo et al., 2014). Indeed, the S1R agonist pridopidine has been shown to rescue a deficit in TTX-induced synaptic scaling in YAC128 cultured cortical neurons, and this effect is reproduced by another S1R agonist, 3-PPP (Smith-Dijak et al., 2019). Moreover, pridopidine and other S1R agonists have been shown to normalise aberrant ER calcium homeostasis in YAC128 MSNs (Ryskamp et al., 2017). Given these findings, studying
pridopidine’s potential therapeutic benefit in HD may elucidate mechanistic features of other neurodegenerative diseases.

1.8. Overall Objective

As previously discussed, one feature of mouse models of HD is the dysregulation of calcium at the level of the ER, whereby mutant HTT directly interacts with and sensitises the type I IP3R to IP3, leading to ER calcium store depletion and causing excessive SOCR. Interestingly, activating S1R has been shown to normalise these aberrant features of ER calcium dynamics in HD (Ryskamp et al., 2017). Another important feature of HD is an increase in extrasynaptic NMDA receptor numbers and function at HD cortical-striatal synapses, which also alters calcium signalling. However, it is unclear how mutant HTT mediates overexpression of extrasynaptic NMDA receptors. Recent work has shown that synaptic NMDA receptor stimulation of the expression of Activin A, a member of the transforming growth factor β (TGF-β) family whose transcription is nuclear-Ca2+-dependent, reduces toxic extrasynaptic NMDA receptor signalling in the hippocampus (among other beneficial effects) (Lau et al., 2015). The ER is suggested to be involved in synapse-to-nuclear calcium signalling (necessary for calcium-dependent transcription of Activin A and other pro-survival factors), while ER calcium handling and CREB signalling are altered in HD. I therefore hypothesise that ER calcium depletion impedes normal synapse-to-nuclear calcium signalling in HD, disrupts activity-dependent gene transcription, and ultimately reduces Activin A expression. I further hypothesise reduced Activin A expression contributes to overexpression of extrasynaptic NMDA receptors in HD, and that S1R agonists can normalise this through restoring synapse-to-nucleus signalling and normalising Activin A levels (Figure 1.3).
**A) Hypothesized pathophysiology of HD**

1. Overactive IP3 receptor due to mHTT
2. Depleted ER Ca\(^{2+}\) stores & ↓Synapse-Nucleus Signaling
3. ↓Ca\(^{2+}\)-Dependent Transcription & ↓Activin A
4. ↑eNMDAR

**B) Hypothesized effect of S1R agonists and Activin A**

1. S1R agonist Normalizes Calcium Store Depletion & Restores Synapse-Nucleus Signaling
2. Intact Ca\(^{2+}\)-Dependent Transcription & Normalized Activin A
3. Normalized eNMDAR

**Figure 1.3: Cartoon diagram depicting my hypothesis.** A) Hypothesised pathology of HD: ER calcium depletion impedes normal synapse-to-nuclear calcium signalling in HD, disrupts activity-dependent gene transcription, and ultimately reduces Activin A expression, leading to overexpression of extrasynaptic NMDA receptors. B) Hypothesised effect of S1R agonists and Activin A: S1R agonists normalise synapse-to-nucleus signalling leading to normalised Activin A levels and reduced extrasynaptic NMDA receptors in HD.
Aim 1 (Chapter 2): Determine if calcium signalling to the nucleus is impaired in HD and explore mechanisms contributing to this deficit.

Aim 2 (Chapter 3): Investigate a therapeutic role for Sigma-1 Receptor and Activin A in normalising synaptic function, including NMDA receptor synaptic/extrasynaptic activity and cytosol to nuclear calcium signalling.

Aim 3 (Chapter 4): Assess the effectiveness of treatment of HD mice with Activin A AAV for improving motor learning and normalising extrasynaptic NMDA receptors in acute slices.
Chapter 2: Calcium signalling to the nucleus is impaired in HD and new mechanisms of synapse-to-nucleus calcium transmission in MSNs have been uncovered

2.1. Introduction

HD is a fatal hereditary neurodegenerative disorder. It is caused by a CAG trinucleotide repeat expansion in the gene coding the protein huntingtin (HTT), resulting in a polyglutamine repeat expansion near the N-terminus of this protein ("A Novel Gene Containing a Trinucleotide Repeat That Is Expanded and Unstable on Huntington’s Disease Chromosomes. The Huntington's Disease Collaborative Research Group," 1993). More than 35 repeats lead to HD, and the age of onset is inversely proportional to the number of CAG repeats, which encode an expanded polyglutamine (polyQ) in HTT (Langbehn et al., 2010).

Dysregulation of synaptic function and calcium handling are common characteristics in HD (Mackay et al., 2018) as well as many other neurodegenerative disorders such as Alzheimer’s (Calderon-Garcidueñas & Duyckaerts, 2017; Liang et al., 2015; Small, 2009; Supnet & Bezprozvanny, 2010) and Parkinson’s (Poewe et al., 2017; Verma et al., 2018; Zaichick et al., 2017). One feature of mouse models of HD is the dysregulation of calcium at the level of the ER, whereby mutant HTT directly interacts with and sensitises IP3R1, (a calcium channel at the ER membrane) to IP3, leading to ER calcium store depletion and causing excessive calcium store refilling (Ryskamp et al., 2017). The IP3R is a calcium channel located on the ER membrane that plays an important role in neuronal calcium signalling. Of the three isoforms, IP3R1 is the most common type found in neurons (Egorova & Bezprozvanny, 2018). The sensitization of IP3R1 to IP3 leads to excessive calcium release from the ER and thus calcium store depletion. This triggers the store-operated calcium response (SOCR) which is the refilling of ER calcium stores using...
plasma membrane calcium channels (I. Bezprozvanny, 2011). Excessive ER calcium release mediated by IP3R1 thus causes excessive calcium store refilling (Geva et al., 2016; Vigont et al., 2015) leading to altered calcium dynamics.

Another important feature of HD is an increase in extrasynaptic NMDA receptor numbers and function at HD cortical-striatal synapses (Milnerwood et al., 2010, 2012; Raymond, 2017), which also alters calcium signalling. Extrasynaptic NMDA receptor activation – due to excess glutamate which also activates NMDA receptors at extrasynaptic sites – opposes plasticity and promotes cell death pathways (Hardingham et al., 2002; Hardingham & Bading, 2010). Calcium entry through synaptic NMDA receptors leads to the activation of calcium/calmodulin-dependent protein kinase IV (CaMKIV), which then phosphorylates and activates the transcription factor CREB at site Ser133 (Hardingham et al., 2001; Hardingham & Bading, 2010; Sun et al., 1994), leading to the transcription of prosurvival genes such as BDNF which has neuroprotective properties (Hansen et al., 2004; Thoenen et al., 1987). In contrast, extrasynaptic NMDA receptor activation leads to CREB dephosphorylation via the juxtasynaptic attractor of caldendrin on dendritic boutons protein (Jacob) (Hardingham & Bading, 2002). Though an increase in extrasynaptic NMDA receptor numbers and function is a prominent HD feature, it is unclear how mutant HTT mediates overexpression of extrasynaptic NMDA receptors. Recent work has shown that Activin A, a member of the transforming growth factor β (TGF-β) family whose transcription is nuclear-calcium-dependent (Bloise et al., 2019), reduces toxic extrasynaptic NMDA receptor signalling in the hippocampus (Lau et al., 2015). Taken together, I hypothesise that synapse-to-nuclear signalling is altered in HD. To study this, I used cortical-striatal co-cultures and transfected the MSNs with nuclear localised GCaMP and a cytosolic localised RCaMP to better study the transfer of calcium from cytosol to nucleus.
2.2. Methods

2.2.1. Cortical-Striatal Cultures and Nucleofections

A colony of FVB/N WT and homozygous YAC128 (line 55) mice (Slow et al., 2003) were bred and maintained in the University of British Columbia Preclinical Discovery Centre according to guidelines of the Canadian Council on Animal Care, under the approved protocol A17-0295. Cortical and striatal neurons were isolated from mouse pups (E17-18) as previously described (Milnerwood et al., 2012). 1 million cortical cells and 1 million striatal cells were suspended in 6 mL D minimum essential medium (DMEM, GIBCO) with 10% foetal bovine serum (DMEM+). Before plating, the 1 million striatal cells were suspended in 100 μL of electroporation buffer (Mirus Bio) with plasmids containing genetically-encoded calcium indicators (GECIs). These GECIs were either a cytosolic GCaMP (Syn-jGCaMP7f-WPRE; Addgene) or both a cytosol-localised RCaMP (Syn.NES-jRCaMP1b.WPRE.SV40; Addgene) and a nucleus-localised GCaMP (CKII.GCaMP3-NLS-myc; gift from H. Bading, Heidelberg U) simultaneously. This solution was placed in a cuvette, electroporated (AMAXA nucleofector I: program 05), and resuspended in 6 mL DMEM+ with 1 million non-transfected cortical cells. Cells were plated at a density of 1.125×10⁵ cells per cm² in three 4-well petri dishes (Cellvis, D35C4-20-1.5-N). After 3 hours, DMEM+ was replaced with 0.5 mL of neurobasal medium (NBM: 2% B27, Invitrogen; penicillin/streptomycin; 2 mM α-glutamine; neurobasal medium A, GIBCO). An additional 0.5 mL/well of NBM was added at 3 days in vitro (DIV), and then half of the culture’s NBM was replaced at DIV10.
2.2.2. Calcium Imaging

Striatal neurons in cortical-striatal co-cultures at DIV 17-19 were imaged in artificial cerebrospinal fluid (ACSF) with the Zeiss Axio Observer Z1, focusing on the soma and the nucleus using a 63x objective in order to optimise for a high signal-to-noise ratio at 10 Hz imaging frequency. For the cultures nucleofected with both cytosol-localised RCaMP and nucleus-localised GCaMP, each channel was imaged one after the other in alternating fashion using LEDs. to obtain an imaging frequency of 10 Hz for both red and green channels, the LEDs would alternate at a rate of 20 Hz. For the cultures nucleofected with the cytosolic GCaMP alone, the field of view contained the soma and up to a 70 μm radius of the dendritic tree so that calcium at the soma and dendrites could be imaged simultaneously. In all instances of calcium imaging, the intensity of the excitation light was adjusted so that only 5% of the dynamic range was utilised when measured at baseline calcium to reduce phototoxicity. For the cultures nucleofected with both cytosol-localised RCaMP and nucleus-localised GCaMP, spatial binning of 2x2 pixels was done during recording to further reduce the excitation light intensity to minimise phototoxicity and photobleaching. Cells were imaged at room temperature.

2.2.3. Drug and Antisense Oligonucleotide (ASO) Treatments

To assess the impact of different calcium channels on spontaneous activity, various pharmacological treatments were applied directly to the wells during calcium imaging. After 2-3 minutes of imaging spontaneous baseline activity, ryanodine (30 μM) was applied to test the effect of blocking RyR on nuclear calcium signalling. The cell was then imaged 5 minutes after RyR application to allow for proper blockade of RyR. (S)-MCPG (250 μM) or vehicle was applied 1 hour in the incubator before recording; (S)-MCPG is an antagonist of metabotropic glutamate
receptor type 1/5 (mGluR1/5) which upon activation by glutamate binding is responsible for the production of IP3 which binds IP3R1. By blocking mGluR1/5, this prevents the production of IP3, and the 1-hour incubation is to ensure that any remaining IP3 was metabolised. To further study the impact of IP3R1 on nuclear calcium signalling, cultures were incubated for 6d with 500 nM antisense oligonucleotide (ASO) against IP3R1 or control ASO (ISIS 536178 and ISIS 676630, respectively; Ionis Pharm.) in the medium, as in (J. Wu et al., 2016). Calcium flow through L-type CaV channels has been reported to have preferential access to the nucleus given these channels’ localization on the somatic plasma membrane (Ahlijanian et al., 1990; Bading, 2013; Westenbroek et al., 1990). To study the contribution of L-type CaV channels on nuclear calcium signalling, nifedipine (10 μM) – an L-type CaV channel blocker – was applied directly to the wells after 2-3 minutes of imaging baseline spontaneous activity. Finally, D-APV (25 μM) – an NMDA receptor blocker – was applied after 2-3 minutes of imaging baseline spontaneous activity to study the contribution of NMDA receptor on nuclear calcium signalling.

2.2.4. Data Analysis and Statistics

Images were analysed with Python code for automatic placement of regions of interest (ROIs) using machine learning, calculation of ΔF/F0, and extraction of trace parameters such as timing, frequency, and amplitude of calcium peaks, as well as total area under the curve (AUC) of these peaks. For analysis of cultures nucleofected with jGCaMP7f, Ilastik – a tool for image classification and segmentation (https://www'ilastik.org/index.html) – was used with Python to train the algorithm to accurately detect the soma and the dendritic tree as 2 separate ROIs (Figure 2.1A). For analysis of cultures nucleofected with both cytosol-localised RCaMP and nucleus-localised GCaMP, a machine learning algorithm was also used to place a nuclear ROI and a cytosolic ROI in the green and red channel respectively (Figure 2.1B). For the cytosol-localised
RCaMP and nucleus-localised GCaMP images, an amplitude ratio of time-locked events as well as an AUC ratio (GCaMP/RCaMP) was computed to assess the nuclear calcium signal/unit of cytosolic calcium to assess the relative efficiency of calcium transfer from the soma to the nucleus. Of note, the relative nuclear and cytosolic calcium signals cannot be directly compared because:

a) different GECIs were used in each respective compartments though GCaMP3 and jRCaMP1b have similar ranges of calcium concentrations that they can detect (Akerboom et al., 2012; Dana et al., 2016) and b) even with the same calcium indicator in both compartments, it has been shown the fluorescence intensity can be higher in the nucleus (H. Nakazawa & Murphy, 1999). For analysis, t-tests or two-way ANOVAs were used to obtain p-values for drug/ASO treatment, genotype, and interactions between the different variables. In each dataset, normality was tested in order to choose between parametric and non-parametric statistical tests. Individual cells rather than culture batch averages were used as biological replicates.
2.3. Results

2.3.1. Cell-wide calcium waves are voltage-gated sodium channel dependent

In order to test whether synapse-to-nuclear signalling is impaired in HD, I transfected striatal cells with a cytosolic GCaMP and applied tetrodotoxin (500 nM) to observe spontaneous calcium waves from dendrites to the soma. The goal was to eliminate the overwhelming cell-wide
calcium waves in order to more readily observe dendrite to soma calcium signals. However, no calcium events were observed in the soma after TTX application (Figure 2.2C and E). This is consistent with CA1 pyramidal neurons as these calcium waves have also been reported not to propagate to the soma (Lee et al., 2016). As a result, spontaneous calcium events in the absence of TTX were studied instead, as calcium events at the soma were readily detectable. Furthermore, these somatic calcium events also occurred synchronously in the dendritic tree in the absence of TTX. These cell-wide calcium events are therefore action potential-dependent.
Figure 2.2: Spontaneous cell-wide calcium events are action potential-dependent. A) Example of an MSN cell expressing jGCaMP7f in a cortical-striatal co-culture with basal calcium (Top) and at the peak of a cell-wide calcium event (Bottom) B) Example ΔF/F0 trace of an ROI placed on the soma showing calcium activity at baseline and after TTX (500 nM) application (greyed area). C) Soma amplitude average before and after TTX application for WT and YAC128 cultures. D) Dendritic amplitude average before and after TTX application for WT and YAC128 cultures. E) Average frequency of calcium events at the soma before and after TTX application for WT and YAC128 cultures. Points represent individual cell averages and error bars represent standard error.
of the mean. P-values for two-way ANOVA analysis are inset within their respective bar graphs (WT, n = 18 cells (4 cultures); YAC128, n = 12 cells (3 cultures)) (BL = Baseline).

2.3.2. Cytosol-to-nucleus calcium signal transmission is reduced in co-cultured YAC128 striatal neurons

In order to test the efficiency of calcium transmission to the nucleus, I transfected MSNs from cortical-striatal co-cultures with a cytosol-localised RCaMP and a nucleus-localised GCaMP. This allows the ability to assess calcium signals in the nucleus and cytosol simultaneously, as opposed to just using a cell-wide GCaMP. Examining spontaneous calcium activity, YAC128 neurons have a smaller average total nuclear AUC than WT (Figure 2.3C; WT: 90.75±5.354; YAC128: 75.94±5.368; p-value: 0.0314), and a smaller GCaMP/RCaMP AUC ratio (Figure 2.3B; WT: 3.305±0.1449; YAC128: 2.826±0.1537; p-value: 0.0197) and amplitude ratio (Figure 2.3E; WT: 3.100±0.1540; YAC128: 2.756±0.1890; p-value: 0.0380). There was no significant genotype effect in the nuclear amplitude (Figure 2.3F) and frequency (Figure 2.3J), nor the cytosolic AUC (Figure 2.3D), amplitude (Figure 2.3G) and frequency (Figure 2.3K). Interestingly, about 18-19% of all cytosolic events did not have a time-locked nuclear event (Figure 2.3H), and the average ΔF/F0 amplitude of these cytosolic events were not different between genotypes and were roughly 0.13-0.15, hinting at a threshold mandatory for entry of calcium into the nucleus. This shows the importance of imaging the nuclear compartment specifically, and not just the soma. For all subsequent figures, I will be looking primarily at the AUC ratios including the total nuclear and cytosolic AUC since the genotype difference lies in those metrics.
**Figure 2.3: Cytosol-to-nucleus calcium signal transmission is reduced in co-cultured YAC128 striatal neurons.** A) Example of an MSN cell (Top: WT, Bottom: YAC128) expressing cytosol-localised RCaMP and nucleus-localised GCaMP at baseline and during a spontaneous event, and example ΔF/F0 traces showing calcium activity in the nucleus (green trace) and cytosol (red trace). B) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs. C) Nuclear total AUC/min average of WT and YAC128 MSNs. D) Cytosol total AUC/min average of WT and YAC128 MSNs. E) Amplitude ratio (average of individual amplitudes of nuclear events/amplitudes of time-locked cytosolic events) average of WT and YAC128 MSNs. F) Nuclear amplitude average of WT and YAC128 MSNs. G) Cytosol amplitude average of events with a time-locked nuclear event of WT and YAC128 MSNs. H) Average fraction of cytosolic events with no time-locked nuclear events for WT and YAC128 MSNs. I) Cytosol amplitude average of events with no time-locked nuclear event of WT and YAC128 MSNs. J) Average nuclear frequency of events of WT and YAC128 MSNs. K) Average cytosolic frequency of events of WT and YAC128 MSNs. Points represent individual cell averages and error bars represent standard error of the mean. P-values for Mann-Whitney t tests are below their respective bar graphs (WT, n = 179 cells (16 cultures); YAC128, n = 139 cells (14 cultures)).

### 2.3.3. Cytosolic and Nuclear calcium signals are greatly decreased after 25 μM D-APV treatment

NMDA receptors are the primary synaptic calcium channel stimulated by glutamate release, and thus assessing their role in synapse-to-nucleus calcium signalling is imperative. To study their contribution in cytosol to nucleus calcium signalling, NMDA receptors were blocked with D-APV (25 μM) after a 2-3 minute baseline recording. In all culture preparations (cytosolic GCaMP alone – Figure 2.4A,B; or both cytosol-localised RCaMP and nucleus-localised GCaMP – Figure 2.4C-F), D-APV eliminated the occurrence of action potential-dependent cell-wide calcium waves in both genotypes (Figure 2.4), which rendered any measurement of nuclear/cytosolic AUC impossible. The complete abrogation of GCaMP- and RCaMP- measurable calcium signals in nucleus and cytosol, respectively, after D-APV confirms the importance of
NMDA receptors as necessary for initiating these action potential-dependent cell-wide calcium waves.

Figure 2.4: Cytosolic and Nuclear calcium signals are greatly decreased after 25 μM D-APV treatment. A-B) Imaging from MSNs expressing cytosolic GCaMP only A) Example ΔF/F0 trace of an ROI placed on the soma showing calcium activity at baseline and after D-APV (25 μM) application. B) Average event amplitude at the soma for WT and YAC128 MSNs before and after D-APV. C-F) Imaging from MSNs expressing nuclear-localized GCaMP and cytosol-localized RCaMP. C) Example ΔF/F0 traces showing calcium activity in the nucleus (green trace) and cytosol (red trace) before and after D-APV treatment. D) Nuclear total AUC/min average of WT and YAC128 MSNs before and after D-APV. E) Cytosol total AUC/min average of WT and YAC128 MSNs before and after D-APV. F) Average nuclear frequency of events of WT and YAC128 MSNs before and after D-APV. Points represent individual cell averages and error bars represent standard error of the mean. P-values for Mann-Whitney t tests comparing genotypes are shown above their respective bar graphs (For B: WT, n = 18 cells (3 cultures); YAC128, n = 8 cells (2 cultures)) (For D, E, and F: WT, n = 23 cells (4 cultures); YAC128, n = 16 cells (3 cultures)) (BL = Baseline).
2.3.4. Cytosolic, but not nuclear calcium signals are decreased after 10 μM Nifedipine treatment

As stated in section 2.2.3, calcium flow through L-type CaV channels has been reported to have preferential access to the nucleus given their localization on the plasma membrane on the soma. To study the contribution of L-type CaV channels on nuclear calcium signalling, nifedipine (10 μM) was applied directly to the wells after 2 minutes of imaging baseline spontaneous activity. Using cytosolic GCaMP, in both genotypes, nifedipine significantly decreases the event amplitude average by ~50% (P = 0.0041 for WT and 0.0231 for YAC128) (Figure 2.5B), but has no significant effect on the frequency of the cell-wide calcium waves (Figure 2.5C and D). The dataset for the cytosol-localised RCaMP and nucleus-localised GCaMP transfected cells mirror that of the cytosolic GCaMP transfected cells. The cytosolic total AUC average significantly decreased by ~60% after nifedipine (P = 0.0076 for WT and 0.0009 for YAC128) (Figure 2.5I), and the frequency of events was not significantly changed (Figure 2.5F). Interestingly, the nuclear total AUC average did not significantly decrease after nifedipine treatment for either genotype (P = 0.1594 for WT and 0.3535 for YAC128). This is also reflected in the significantly increased average AUC ratio by ~100% (P = 0.023 for WT and 0.0219 for YAC128). This suggests that L-type CaV channels may have less of a role in nuclear calcium signalling in MSNs compared to other cell types such as hippocampal CA1 pyramidal neurons (Bengtson & Bading, 2012). The p-values in this section were determined by paired t tests for before/after drug within genotype.
Figure 2.5: Cytosolic, but not nuclear calcium signals are decreased after 10 μM Nifedipine treatment. A-D) Imaging from MSNs expressing cytosolic GCaMP only. A) Example ΔF/F0 trace of an ROI placed on the soma showing calcium activity at baseline and after nifedipine (10 μM) application. B) Average event amplitude at the soma for WT and YAC128 MSNs before and after nifedipine. C) Average event frequency at the soma for WT and YAC128 MSNs before and after nifedipine. D) Average event frequency at the dendritic tree for WT and YAC128 MSNs before and after nifedipine. E-I) Imaging from MSNs expressing nucleus-localised GCaMP and cytosol-localised RCaMP. E) Example ΔF/F0 traces showing calcium activity in the nucleus (green trace) and cytosol (red trace) before and after nifedipine treatment. F) Average nuclear frequency of events of WT and YAC128 MSNs before and after nifedipine. G) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs before and after nifedipine. H) Nuclear total AUC/min average of WT and YAC128 MSNs before and after nifedipine. I) Cytosol total AUC/min average of WT and YAC128 MSNs before and after nifedipine. Points represent individual cell averages and error bars represent standard error of the mean. P-values above their respective bar graphs are
for unpaired t tests comparing genotypes (For B, C, and D: WT, n = 11 cells (2 cultures); YAC128, n = 13 cells (3 cultures)) (For F, G, H, and I: WT, n = 17 cells (4 cultures); YAC128, n = 9 cells (2 cultures)) (BL = Baseline).

2.3.5. Cytosolic and nuclear calcium signals are unaffected after 250 μM (S)-MCPG pre-treatment or after IP3 knock-down using an ASO

Given that IP3R1 are more sensitised to IP3 in HD and that consequently, IP3 receptors are open more chronically (Ryskamp et al., 2017), I hypothesised that blocking IP3 receptors should restore CICR and normalise the decreased GCaMP/RCaMP AUC ratio in YAC128 MSNs. To test this, I used the mGluR1/5 antagonist MCPG (250 μM). As I stated in the methods (section 2.2.3), MCPG leads to the halting of IP3 production. Unlike the other pharmacological experiments in this chapter, these data are unpaired due to the one hour minimum incubation time with MCPG to ensure all the basal IP3 is metabolised. Comparing the control to the MCPG treated cells, there was no observed effect of MCPG on GCaMP/RCaMP AUC ratio (Figure 2.6A), nuclear total AUC/min (Figure 2.6B), and cytosolic total AUC/min (Figure 2.6C).

To target IP3 receptors more directly, I used an ASO against IP3R1 or control ASO (ISIS 536178 and ISIS 676630, respectively; Ionis Pharm.). However, the data mirrors that of the MCPG data. One caveat is that although we used the same concentration and time course for the ASO treatment as in the literature (J. Wu et al., 2016), a western blot is needed to assess successful knock-down of the IP3R1. However, taken together, IP3R1 seems to play a minimal role in action potential-dependent cytosolic or nuclear calcium signalling.
Figure 2.6: Cytosolic and nuclear calcium signals are unaffected after 250 μM (S)-MCPG pre-treatment or after IP3 KD using an ASO. A) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs with and without MCPG. B) Nuclear total AUC/min average of WT and YAC128 MSNs with and without MCPG. C) Cytosol total AUC/min average of WT and YAC128 MSNs with and without MCPG. D) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs with a control ASO (ASO Ctrl) or an ASO targeting IP3R1 (ASO IP3). E) Nuclear total AUC/min average of WT and YAC128 MSNs with a control ASO or an ASO targeting IP3R1. F) Cytosol total AUC/min average of WT and YAC128 MSNs with a control ASO or an ASO targeting IP3R1. Points represent individual cell averages and error bars represent standard error of the mean. P-values for two-way ANOVA tests are above their respective bar graphs (For A, B, and C: WT Control, n = 29 cells (3 cultures); WT MCPG, n = 34 cells (3 cultures); YAC128 Control, n = 15 cells (2 cultures); YAC128 MCPG, n = 21 cells (2 cultures)) (For D, E, and F: WT ASO Ctrl, n = 33 cells (3 cultures); WT ASO IP3, n = 31 cells (3 cultures); YAC128 ASO Ctrl, n = 26 cells (3 cultures); YAC128 ASO IP3, n = 24 cells (3 cultures)).
2.3.6. Cytosolic and nuclear calcium signals are reduced after 30 μM Ryanodine treatment

Given the decreased GCaMP/RCaMP AUC ratio in YAC128, I tested whether deceased ER calcium stores might be an underlying factor. To do this, I used high dose ryanodine (30 μM) to block RyR. Briefly, I imaged the cell for baseline activity for 150 seconds, and I then applied ryanodine into the well. I waited 5 minutes for ryanodine to successfully block RyRs, and did not image during these 5 minutes to minimise phototoxicity. I then proceeded to image the cell after ryanodine application.

I expected a greater decrease in the GCaMP/RCaMP AUC ratio in WT MSNs versus YAC128 MSNs after blocking RyRs. To begin, the cytosolic GCaMP data shows a significant 70-80% decrease in amplitude of calcium events at the soma for both WT and YAC128 MSNs (P = 0.0016 for WT and 0.0035 for YAC128) (Figure 2.7B), and a significant decrease in frequency of calcium events at the soma (P = 0.0077 for WT and 0.0002 for YAC128) (Figure 2.7C), but not at the dendrites (Figure 2.7D). For the cytosol-localised RCaMP and nucleus-localised GCaMP data, frequency of events at the nucleus was significantly decreased by ~60% in both genotypes (P = 0.0010 for WT and 0.0105 for YAC128) (Figure 2.7F), and both total nuclear (P = 0.0049 for WT and 0.0009 for YAC128) and cytosolic (P = 0.0047 for WT and <0.0001 for YAC128) AUCs were significantly decreased by ~60% in both genotypes as well (Figures 2.7H and 2.7I). Given this equal drop in both nuclear and cytosolic AUC, the GCaMP/RCaMP AUC ratio unsurprisingly was unchanged after high dose ryanodine (Figure 2.7G). However, there is a trend toward decreased GCaMP/RCaMP ratio in WT (P = 0.0562) but not in YAC128 (P = 0.9473) cultures; a larger sample size may be needed for the difference to be significant. Although no genotype difference was observed, these data highlight the importance of ER calcium stores in nuclear calcium
signalling, and also show the differential impact of these stores on spontaneous action potential-induced calcium activity in the dendritic tree vs. the soma. The p-values in this section were determined by paired t tests for before/after drug within genotype.

Figure 2.7: Cytosolic and nuclear calcium signals are reduced after 30 μM Ryanodine treatment. A-D) Imaging from MSNs expressing cytosolic GCaMP only. A) Example ΔF/F0 trace of an ROI placed on the soma showing calcium activity at baseline and 5 minutes after ryanodine (30 μM) application. B) Average event amplitude at the soma for WT and YAC128 MSNs before and after ryanodine. C) Average event frequency at the soma for WT and YAC128 MSNs before and after ryanodine. D) Average event frequency at the dendritic tree for WT and YAC128 MSNs before and after ryanodine. E-I) Imaging from MSNs expressing nucleus-localised GCaMP and cytosol-localised RCaMP. E) Example ΔF/F0 traces from WT MSNs showing calcium activity in
the nucleus (green trace) and cytosol (red trace) before (baseline) and after 5 minutes ryanodine treatment. F) Average nuclear frequency of events of WT and YAC128 MSNs before and after ryanodine. G) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs before and after ryanodine. H) Nuclear total AUC/min average of WT and YAC128 MSNs before and after ryanodine. I) Cytosol total AUC/min average of WT and YAC128 MSNs before and after ryanodine. Points represent individual cell averages and error bars represent standard error of the mean. P-values above their respective bar graphs are for unpaired t tests comparing genotypes (For B, C, and D: WT, n = 22 cells (4 cultures); YAC128, n = 22 cells (4 cultures)) (For F, G, H, and I: WT, n = 18 cells (3 cultures); YAC128, n = 17 cells (3 cultures)) (BL = Baseline).

2.4. Discussion

In this chapter, I used calcium indicators to explore synapse-to-nucleus signalling in an HD mouse model. To do so, I used cortical-striatal co-cultures. In the presence of TTX, I was able to observe calcium waves, but none that could propagate all the way to the soma. This is consistent with CA1 pyramidal neurons as these calcium waves have also been reported not to propagate very far (Lee et al., 2016). Given the absence of detectable calcium signals at the soma in the presence of TTX, I began to study spontaneous action potential-dependent calcium signals using a novel calcium imaging paradigm whereby I imaged both nuclear and cytosolic calcium simultaneously with the aid of a nucleus-localised GCaMP and a cytosol-localised RCaMP. I will hereby refer to this calcium imaging technique as dual-channel imaging.

A common assumption in the literature is that any calcium event observed at the soma is also reflected in the nucleus. This is due to the high permeability of nuclear pore complexes to calcium. However, some studies suggest there are differences between nuclear calcium versus cytosolic calcium dynamics (Gerasimenko & Gerasimenko, 2004; Martins et al., 2016). It is also for this reason that I opted to image both nuclear and cytosolic compartments simultaneously to
be more certain that calcium observed events indeed happen in the nucleus and not just at the level of the cytosol at the soma.

Using dual-channel imaging, I found that a ratio of the area under the curve (AUC) of the nuclear $\Delta F/F_0$ trace over the cytosolic $\Delta F/F_0$ trace yielded a smaller ratio for YAC128 MSNs compared to WT MSNs. The nuclear calcium transient largely stems from the cytosolic calcium transient, and the main source of these cytosolic transient elevations is calcium release from intracellular stores and influx from the extracellular space through calcium-permeable channels. Therefore, this decreased ratio suggests that there is a deficit in the transfer of calcium from cytosol to nucleus in YAC128 MSNs. This is a novel finding that is supported by decreased calcium-dependent gene transcription found in YAC128 mice (Bayram-Weston et al., 2015). In fact, the nuclear AUC in response to action potential-dependent calcium transients is also significantly smaller in YAC128 MSNs despite the cytosolic AUC not being significantly different between the genotypes. The frequency of nuclear and cytosolic events however are the same for both genotypes, which implies that the calcium signalling deficit to the nucleus observed in YAC128 MSNs is largely due to a problem of calcium transfer into the nucleus, and not a problem of fewer calcium events. Interestingly, some cytosolic calcium events do not have a corresponding time-locked nuclear event. In fact, about 20% of cytosolic events happen exclusively in the cytosol at the soma and not at the nucleus. The amplitudes of these cytosolic events are much smaller than those with a corresponding time-locked nuclear event ($\sim 0.15 \Delta F/F_0$ vs $\sim 0.35 \Delta F/F_0$) for both genotypes. This hints at a defined calcium concentration threshold that must be exceeded in the cytosol at the soma before achieving access to the nuclear space. Perhaps the nuclear envelope acts as a diffusion barrier. GCaMP3 – the genetically encoded calcium indicator I used for nuclear calcium imaging – has very similar, if not higher sensitivity (Tian et al., 2009) to calcium than the
jRCaMP1b used in the cytosol (Dana et al., 2016). Furthermore, the GCaMP3 was also brighter than the jRCaMP1b when comparing their ΔF/F0 values. Taken together, this suggests that GCaMP3 in the nucleus should be able to detect any calcium event that is also readily detected by jRCaMP1b in the cytosol; this further supports the conclusion that not all cytosolic calcium events are reflected in the nucleus, underscoring the need for a nuclear calcium indicator to accurately detect nuclear calcium activity.

I next explored the contribution of NMDA receptors on nuclear calcium signalling. These calcium-permeable receptors open in response to presynaptic glutamate release coupled with postsynaptic depolarization, mediated mainly by AMPA receptors. Thus, synaptic activation by glutamate contributes to nuclear calcium signalling by direct, NMDA receptor-mediated influx of calcium to the cytosol and by depolarization-induced generation of action potentials and opening of L-type CaV channels (Bading, 2013; Bengtson & Bading, 2012; Karpova et al., 2012). I therefore expected to observe only a small decrease in nuclear calcium signalling following NMDA receptor blockade, since AMPA receptors are still active at the synapse to trigger depolarization-induced action potentials. However, the effect on nuclear calcium signalling by blocking NMDA receptors was robust. Using the NMDA receptor antagonist D-APV, I observed the elimination of action potential-dependent cell-wide calcium waves in both nuclear and cytosolic compartments and in both genotypes. This positions NMDA receptors as an essential initiator of activity-dependent nuclear calcium signalling.

Calcium flow through L-type CaV channels has been reported to have preferential access to the nucleus given these channels’ localization on the somatic plasma membrane (Bading, 2013; Westenbroek et al., 1990). Therefore, I expected blocking these channels would markedly decrease nuclear calcium signalling in MSNs. However, I did not observe a significant decrease in nuclear
calcium event frequency nor nuclear AUC after nifedipine application. Interestingly, a very significant and large drop of ~70% was observed in the cytosolic AUC. This led to a significant increase in the nuclear to cytosol AUC ratio (GCaMP/RCaMP). In the literature, most studies reporting the contribution of L-type CaV channels use a cytosolic calcium indicator in the soma. Indeed, this is consistent with the drop in cytosolic calcium AUC I observed. However, the differing effect of nifedipine on nuclear calcium AUC further stresses the importance of using a nuclear-localised calcium indicator to study action potential-induced nuclear calcium transients. Of note, this apparently insignificant role of L-type CaV channels in transmitting calcium signals from cytosol to nucleus may be specific to MSNs, as most studies exploring nuclear calcium dynamics are done with excitatory CA1 hippocampal neurons.

With a nuclear calcium signalling deficit in YAC128 MSNs established, I aimed to find the source of this difference in calcium signalling. One feature of mouse models of HD is the dysregulation of calcium at the level of the ER, reviewed in (Mackay et al., 2018), whereby mutant HTT directly interacts with and sensitises the type 1 inositol (1,4,5)-trisphosphate receptor (IP3R1, a calcium channel at the ER membrane) to IP3 (I. Bezprozvanny, 2011). I therefore investigated the effect of IP3 receptors on nuclear calcium signalling. Due to the calcium leak through IP3 receptors, I hypothesised that preventing calcium efflux through them would restore the AUC ratio (GCaMP/RCaMP) of YAC128 MSNs to that of the WT MSNs. Ultimately, no significant effect was detected on AUC ratio (GCaMP/RCaMP), nuclear AUC, or cytosolic AUC after treatment with MCPG (mGluR1/5 antagonist, which should reduce cytosolic levels of IP3) or the IP3R1 ASO. As previously mentioned, a western blot is needed to verify knockdown of the IP3 receptor. However, taken together, IP3R1 seems to play a minimal role in action potential-dependent cytosolic or nuclear calcium signalling. As stated in chapter 1, IP3 receptors typically are only
open very briefly, and the observed calcium transients are also very brief (4-9 ms) (Shuai et al., 2007); this could explain the minimal contribution of IP3 receptors to nuclear calcium signalling (Berridge, 2016; Taylor et al., 2004). However, a more effective way to reduce IP3 from all upstream sources (i.e., the many receptors/signalling pathways that increase IP3 levels) would be to inhibit phospholipase C which should be done in future experiments.

Interestingly, RyRs are also leaky in HD (Suzuki et al., 2012). Therefore, I hypothesised that ER calcium stores were depleted in YAC128, and that removing CICR by blocking RyR may decrease nuclear calcium signalling more in WT than in YAC128 MSNs. Instead, I found that blocking RyR led to a significant decrease in nuclear and cytosolic AUC to the same extent for both genotypes. Furthermore, this decrease was approximately proportional in both genotypes as the AUC ratio (GCaMP/RCaMP) did not significantly change, although there was a trend toward a reduction in this ratio for WT which could be further explored by repeating these experiments in additional culture batches. Interestingly, the frequency of events was also decreased except for the frequency of cell-wide calcium events in the dendritic tree (imaged with cytosolic GCaMP). These results suggest that ryanodine receptors have greater importance at the soma than the dendritic tree. However, the amplitude of these cell-wide calcium waves was also decreased in the dendritic tree. What may account for the differing effects between the soma and the dendrites is that the soma has a bigger volume than individual dendrites, and so without the amplification effect of CICR, calcium events may not be as readily detectable at the soma compared to dendrites. Though no significant genotype difference could be detected with high dose ryanodine application, this finding places RyRs as important in cytosol to nuclear signalling in MSNs.

Taken together, I found a deficit in nuclear calcium signalling in YAC128 MSNs, but could not isolate the source of that difference. Perhaps the dual channel imaging requires a larger sample
size due to the variability from one MSN to the next. However, these cultures are from embryonic day 17 mice, and so this is very early in the time course of HD. The presence of a deficit at this age magnifies the importance of early treatment of HD. Even though the mechanism(s) underlying the observed nuclear calcium signalling deficits in YAC128 MSNs could not be definitively identified, new insights into calcium signalling to the nucleus in MSNs were uncovered. NMDA receptors seem to be the key initiator of action potential-dependent cell-wide calcium waves to the nucleus. This signal is then magnified by RyR-mediated CICR, leading to a nuclear calcium signal. Calcium influx via L-type CaV channels may be important in binding calmodulin in the soma, which is then trafficked to the nucleus where it can regulate transcription (Barreda et al., 2011; Huang & Huang, 2011). This may explain the lack of an effect on nuclear calcium signalling observed with inhibition of L-type CaV channels. Furthermore, the differing effect of nifedipine on nuclear and cytosolic calcium AUC implies a different source of calcium for the nucleus than simply the passive transmission of cytosolic calcium transients to the nucleus. The outer nuclear membrane also contains RyR, which may be able to activate fully in response to the residual cytosolic calcium, which may be coming from cytosolic ER.

To measure these calcium signals more accurately, a ratiometric calcium indicator would be ideal. With the current dual channel imaging paradigm used in this chapter, it is difficult to observe smaller calcium events in the presence of action potential-dependent cell-wide calcium waves. With the use of a ratiometric calcium indicator, measuring calcium concentration may decrease the variability between different cells. Furthermore, an ER-localised calcium indicator would shed new insight into synapse-to-nucleus signalling as the ER is hypothesised to be depleted of its calcium stores in YAC128 mice. Ratiometric calcium indicators may help elucidate genotype
differences more accurately, which would aid in understanding the underpinnings of altered calcium handling in HD.

Finally, sources of culture variability may be attributed to the fact that these cortical-striatal co-cultures were a mix of cells from male and female embryos. Furthermore, the striatal neurons were a mix of both D1- and D2-expressing MSNs which are both included in the datasets as I did not distinguish them. In future experiments, examining specifically D1- or D2-expressing MSNs may lower the observed variability.
Chapter 3: Activin A is reduced in YAC128 cortical-striatal co-cultures and modulates surface expression of GluN2B-NMDAR in striatal neurons

3.1. Introduction

IP3R1 binds to mutant HTT, and as a result, is more sensitive to and will gate open in response to lower concentrations of IP3 (J. Wu et al., 2016). This sensitivity was reduced back to that of WT after pridopidine treatment, an agonist of S1R (Ryskamp et al., 2017). Furthermore, extrasynaptic NMDA receptors are increased in cortical-striatal synapses (Milnerwood et al., 2010), but the mechanism by which mutant HTT is linked to increased extrasynaptic NMDA receptors is not well understood. I have found that YAC128 MSNs exhibit nuclear calcium signalling deficits as seen by the decreased AUC ratio (nucleus/cytosol) demonstrated in Chapter 2.

Activin A is a dimer of inhibin-β-A subunits that is secreted and binds to plasma membrane receptors belonging to a serine/threonine kinase family known as type I (low molecular weight) and type II (high molecular weight) activin receptors (ACVRs) (Lau et al., 2015). It is expressed ubiquitously and has effects in other systems beyond the nervous system. Most research on Activin A has been done in reproductive physiology, whereby it is involved in the human foetal ovary development as well as the stages of follicular and luteal development (Bloise et al., 2019). Regarding the brain, Activin A has been demonstrated to be neuroprotective. The Bading lab has found that Activin A – a member of the transforming growth factor β (TGF-β) family whose transcription is nuclear calcium-dependent – is able to decrease toxic extrasynaptic NMDA receptor calcium flux (Lau et al., 2015). Specifically, the Bading lab has shown that synaptic NMDA receptor activation leads to nuclear calcium signalling which then promotes the
transcription of Activin A, which is secreted and binds ALK4 (activin-like kinase 4, also known as Activin receptor IB [ACVR1B]). This leads to the reduction of toxic extrasynaptic NMDA receptor calcium influx as evidenced by Fluo-3 calcium imaging experiments using MK-801 to block synaptic NMDA receptors, and in the presence of blockers of voltage-gated calcium channels and action potentials (Lau et al., 2015).

Taken together, I hypothesise that Activin A is decreased in YAC128 cultures, and that this may be rescued by S1R agonists like pridopidine treatment via the normalization of nuclear calcium signalling. I also hypothesise that upon rescuing decreased Activin A with a S1R agonist, this would lead to a decrease in membrane extrasynaptic NMDA receptors. To study this, I used cortical-striatal co-cultures to quantify Activin A in the culture media in WT and YAC128 cortical-striatal co-cultures. Furthermore, I examined the effect of S1R agonists such as pridopidine and 3-PPP on nuclear calcium signalling. Finally, I studied the effect of Activin A and S1R agonists on GluN2B localization (many GluN2B-containing NMDA receptors are preferentially found extrasynaptically).

3.2. Methods

3.2.1. Cortical-Striatal Cultures and Nucleofections

Cortical-striatal cultures were made as described in section 2.2.1. Changes involved the plasmids used for transfections depending on the experiment. For calcium imaging, the combined cytosol-localised RCaMP (Syn.NES-jRCaMP1b.WPRE.SV40; Addgene) and nucleus-localised GCaMP (CKII.GCaMP3-NLS-myc; gift from H. Bading, Heidelberg U) from the previous chapter were once again used. For the Activin A ELISA, the neurobasal medium contained no pH indicator
so that the colour of the media would not interfere with the Activin A ELISA. For the GluN2B surface/internal imaging, MSNs were transfected with GluN2B-YFP (Syn.YFP-GluN2B; gift from A.M. Craig, University of British Columbia; originally from M. Sheng, Genentech, San Francisco). Media changes were done as in section 2.2.1.

3.2.2. Activin A ELISA

Activin A is released into the extracellular space. Due to this, Activin A can be measured in the culture media. Media was taken from WT and YAC128 culture wells at DIV 4 and DIV 21 and stored in -80°C. An Activin A ELISA kit (Human/Mouse/Rat Activin A Quantikine ELISA Kit, R&D Systems, DAC00B) was used to measure Activin A in the media. Total protein concentration was also measured using a protein assay (DC™ Protein Assay Kit II, BIO-RAD, 5000112) in order to normalise Activin A levels to total protein. Measurements at DIV21 were normalised to that of DIV4.

3.2.3. Calcium Imaging

Calcium imaging was done as in section 2.2.2.

3.2.4 GluN2B Surface/Internal Imaging

Surface expression of extrasynaptic GluN2B-containing NMDA receptors is increased in striatal projection neurons in HD, and this overexpression leads to increased striatal neuronal loss in HD mice (Milnerwood et al., 2012). Furthermore, measuring the surface to internal ratio of GluN2B is a proxy for relative extrasynaptic GluN2B-containing NMDA receptor expression (Milnerwood et al., 2012). Thus, given Activin A’s ability to reduce toxic extrasynaptic NMDA receptor calcium flux (Lau et al., 2015), I assessed its effect on surface GluN2B expression by
differential staining of surface and internal GluN2B. To do this, MSN cells from cortical-striatal co-cultures were nucleofected with a GluN2B-YFP construct and plated on coverslips. At DIV21, cells were stained with chicken anti-green fluorescent protein (GFP) antibody (cross reactive with YFP; ab13970, AbCam) for 10 minutes at 37°C, diluted 1:1000 in neurobasal medium at 37°C. Cells were then fixed in 4% paraformaldehyde (PFA) and 2% sucrose for 15 minutes at room temperature. After washing the cells with phosphate buffered saline (PBS), cells were treated with a secondary antibody (Alexa 488 anti-chicken, A11039, Molecular Probes, 1:1000 diluted in PBS) for 1 hour at room temperature and protected from the light on a shaker. Cells were then washed with PBS containing 0.03% Triton X-100 (PBST) to permeabilize the plasma membrane, then stained with chicken anti-GFP antibody (cross reactive with YFP; ab13970, AbCam, 1:1000 diluted in PBST) for 1 hour at room temperature and protected from the light on a shaker. After another round of PBST washing, cells were stained with either anti-chicken Alexa 568 (red fluorescent; A21069, Molecular Probes, 1:1000 diluted in PBST) or anti-chicken AMCA (blue fluorescent; 706-155-148, Jackson Laboratories, 1:1000 diluted in PBST) conjugated secondary antibodies for 1 hour at room temperature and protected from the light on a shaker. Coverslips were then washed with PBST and mounted onto slides using fluoromount G (Southern Biotech). Mounted slides were then imaged with the Zeiss Axio Observer Z1, focusing on the soma and up to a 70 μm radius of the dendritic using a 63x objective in order to optimise for a high signal-to-noise ratio.

3.2.5. Drug and AAV Treatments

To study the effect of S1R agonists on Activin A protein expression, I applied Pridopidine (1 μM) or 3-PPP (1 μM) at DIV10 and assessed Activin A levels using ELISA at DIV 21. I also studied whether pridopidine could ameliorate the genotype difference in the AUC ratio observed
in Figure 2.3. To do this, I assessed 2 different time courses; I applied pridopidine (1 μM) at either DIV 13-15 (4-day treatment) or DIV 16-18 (1-day treatment) before imaging calcium using the combined cytosol-localised RCaMP and nucleus-localised GCaMP (as described in section 2.2.2) at DIV 17-19. To explore the effect of Activin A and SIR agonists on extrasynaptic NMDA receptors, I used the paradigm described in section 3.2.4 and treated cultures with either: 1. An Activin A AAV (rAAV-hSyn-inhba-HA/hSyn-tdimer, gift from H. Bading, Heidelberg U) at a concentration of ~10^9 viral count/mL at DIV 7 (as a control, I used rAAV-CMV/CBA-mCherry NLSr, gift from H. Bading, Heidelberg U); 2. Pridopidine (1 μM) at DIV10; or 3. 3-PPP (1 μM) at DIV 10.

3.2.6. Data Analysis and Statistics

For analysis of the ELISAs, Activin A values were normalised to total protein as measured by DC assay to account for variability in number of cells plated per well. DIV21 data was then normalised to DIV4 data, and a two-way ANOVA was done to explore the effect of days in vitro, and the genotype effect. Activin A ELISAs done with either pridopidine or 3-PPP treatment were analysed in the same way, but the analysis was done only with the normalised DIV21 data to examine both the genotype effect and if there was an overall treatment effect.

As in section 2.2.4, images of cultures nucleofected with both cytosol-localised RCaMP and nucleus-localised GCaMP were analysed with Python code for automatic placement of regions of interest (ROIs) using machine learning, calculation of ΔF/F0, and extraction of trace parameters such as total AUC. The data was then analysed using a two-way ANOVA to explore genotype effects and the pridopidine effect.
For the GluN2B surface/internal images, 3 ROIs were manually placed on different secondary or tertiary dendrites, and a rolling ball filter was applied to subtract the background (Sternberg, 1983). The ratio of the surface GluN2B intensity divided by the internal GluN2B intensity was computed per image, and images were normalised to the control WT average within each culture batch. To be able to normalise within culture batches, all the cells of a single culture batch (WT and YAC128 plated at the same time) were immunostained at the same time, and imaged all on the same day with the exact same excitation light intensities and exposure times per channel. The data was then analysed using a two-way ANOVA to explore both genotype and treatment effects. Individual cells rather than culture batch averages were used as biological replicates.

3.3. Results

3.3.1 Activin A secretion is decreased in YAC128 cortical-striatal co-cultures

To test whether Activin A is decreased in YAC128, I used an Activin A ELISA kit to measure the Activin A concentration in the culture media as described in section 3.2.2. To minimise variability in week-to-week culture handling, WT and YAC128 cultures were plated the same day and normalised within weeks. At DIV21 relative to DIV4, the Activin A concentration was indeed decreased in YAC128 culture media compared to that of WT cultures (Figure 3.1).
Figure 3.1: Activin A secretion is decreased in YAC128 cortical-striatal co-cultures. A) Activin A concentration average in WT and YAC128 culture media presented as a fold increase at DIV21 compared to DIV4. Error bars represent standard error of the mean. B) Points represent Activin A levels in individual cultures at DIV4 and DIV21. P-values for two-way ANOVA analysis are inset in the panel B graph. (WT, n = 9 cultures); YAC128, n = 9 cultures) (DIV = days in vitro).

3.3.2. Pridopidine and 3-PPP have no effect on reduced Activin A secretion in YAC128 MSNs

As stated in my hypothesis, I expect the decreased Activin A observed in YAC128 cultures to be normalised after S1R agonist treatment. To test this hypothesis, I used the Activin A ELISA as described in section 3.2.2, while also treating the cultures at DIV10 with either pridopidine (1 μM) or 3-PPP (1 μM) – two S1R agonists. The data was then normalised to a DIV4 control. At DIV21, I did not observe any effect of pridopidine or 3-PPP on Activin A levels (Figure 3.2). To be certain of the inefficacy of S1R agonists in normalising Activin A levels, more timepoints may be needed.
Figure 3.2: Pridopidine and 3-PPP have no effect on reduced Activin A secretion in YAC128 MSNs. A) Activin A concentration average in WT and YAC128 culture media after either water control, pridopidine (1 μM), or 3-PPP (1 μM) presented as a fold increase at DIV21 compared to DIV4. Error bars represent standard error of the mean. Points represent Activin A levels in individual cultures at DIV21 normalised to DIV4. P-values for two-way ANOVA analysis are above the graph. (WT, n = 5 cultures); YAC128, n = 5 cultures).

3.3.3. Cytosolic and nuclear calcium signals are unchanged after 1 μM pridopidine pretreatment for 1 and 4 days

As has been previously reported, the S1R agonist pridopidine is able to normalise excessive calcium release from sensitised IP3R1 in cultured neurons derived from HD mice (Ryskamp et al., 2017). I hypothesised that S1R agonists should restore CICR – via normalisation of excessive calcium release through sensitised IP3R1 – and normalise the decreased GCaMP/RCaMP AUC ratio in YAC128 MSNs observed in chapter 2 (Figure 2.3). I tested this by applying pridopidine
(1 μM) onto cultures at either DIV 13-15 (4-day treatment) or DIV 16-18 (1-day treatment) before imaging calcium using the combined cytosol-localised RCaMP and nucleus-localised GCaMP (as described in section 2.2.2) at DIV 17-19. After imaging, no significant change in GCaMP/RCaMP AUC ratio, nuclear total AUC, and cytosol total AUC was observed after 1-day (Figure 3.3A-C) or 4-day (Figure 3.3D-F) pridopidine (1 μM) treatment. The 1-day treatment was chosen to mimic the same time course for normalisation of IP3R1 previously reported (Ryskamp et al., 2017). The 4-day treatment was also chosen to match the time course for normalisation of homeostatic synaptic plasticity found in our lab (Smith-Dijak et al., 2019).

Figure 3.3: Cytosolic and nuclear calcium signals are unchanged after 1 μM pridopidine pretreatment for 1 and 4 days. A-C) Imaging from MSNs pretreated 1 day with pridopidine (1 μM). A) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs with and
without pridopidine pretreatment (1 day). B) Nuclear total AUC/min average of WT and YAC128 MSNs with and without pridopidine pretreatment (1 day). C) Cytosol total AUC/min average of WT and YAC128 MSNs with and without pridopidine pretreatment (1 day). D-F) Imaging from MSNs pretreated 4 days with pridopidine (1 μM). D) AUC ratio (nuclear AUC/cytosol AUC) average of WT and YAC128 MSNs with and without pridopidine pretreatment (4 day). E) Nuclear total AUC/min average of WT and YAC128 MSNs with and without pridopidine pretreatment (4 day). C) Cytosol total AUC/min average of WT and YAC128 MSNs with and without pridopidine pretreatment (4 day). Points represent individual cell averages and error bars represent standard error of the mean. P-values for two-way ANOVA tests are above their respective bar graphs (For A, B, and C: WT Control, n = 18 cells (2 cultures); WT Pri (1 day), n = 17 cells (2 cultures); YAC128 Control, n = 15 cells (2 cultures); YAC128 Pri (1 day), n = 22 cells (2 cultures)) (For D, E, and F: WT Control, n = 31 cells (3 cultures); WT Pri (4 days), n = 27 cells (3 cultures); YAC128 Control, n = 30 cells (3 cultures); YAC128 Pri (4 days), n = 34 cells (3 cultures)) (Pri = pridopidine).

3.3.4. Activin A overexpression normalises GluN2B surface expression in YAC128 striatal neurons

Surface expression of GluN2B-containing NMDA receptors is increased in cortical-striatal synapses in HD at extrasynaptic sites, and this overexpression leads to increased vulnerability to striatal neuronal loss in HD mice (Milnerwood et al., 2012). Thus, given Activin A’s ability to reduce toxic extrasynaptic NMDA receptor calcium flux (Lau et al., 2015), I assessed its effect on surface GluN2B expression as described in section 3.2.4. To do this I treated cultures with either an Activin A AAV or a control AAV (nucleus-localised mCherry AAV) at DIV7 as described in section 3.2.5, and then stained the cultures to image surface vs. internal GluN2B as described in section 3.2.4. After imaging and analysis, results show that Activin A overexpression decreased the surface/internal GluN2B ratio in YAC128 MSNs back to that of WT MSNs, but did not decrease the ratio within WT MSNs (Figure 3.4B).
Figure 3.4: Activin A overexpression normalises GluN2B surface expression in YAC128 striatal neurons. A) Example of MSN cells stained for internal (blue) and surface (green) GluN2B. Nucleus-localised mCherry AAV (SHAM) (Top) and Activin A AAV (Bottom) treated cells are in red. Merged channels are displayed to the right of the panel. B) Surface/internal GluN2B average of WT and YAC128 MSNs treated with either nucleus-localised mCherry AAV (SHAM) or Activin A AAV (Act). Points represent individual cell averages and error bars represent standard error of the mean. P-values for two-way ANOVA test are above the bar graph (WT SHAM, n = 26 cells (4 cultures); WT Act, n = 25 cells (4 cultures); YAC128 SHAM, n = 19 cells (4 cultures); YAC128 Act, n = 20 cells (4 cultures)) (Bonferroni multiple comparisons test: SHAM vs. Act: WT p-value = 0.1476; YAC128 p-value = 0.0075).

3.3.5. 3-PPP 1 μM treatment and pridopidine 1 μM treatment may preferentially decrease GluN2B surface expression in YAC128 MSNs vs. WT MSNs

Finally I explored whether S1R agonists could normalise surface GluN2B-NMDA receptor levels in YAC128 MSNs. To test this, I used the GluN2B surface/internal imaging experiment (section 3.2.4) and treated the cultures with either pridopidine (1 μM) at DIV10 or 3-PPP (1 μM) at DIV 10. After imaging and analysis, I observed a trend of a larger decrease in the surface/internal GluN2B ratio in YAC128 MSNs versus WT MSNs after S1R agonist treatment (Figure 3.5C-D).
When using an unpaired t test to compare the individual effects of 3-PPP and pridopidine, however, the genotype difference was not significant. Perhaps a larger sample size is needed.

Figure 3.5: 3-PPP 1 μM treatment and pridopidine 1 μM treatment may preferentially decrease GluN2B surface expression in YAC128 MSNs vs. WT MSNs. A) Imaging from MSNs treated with 3-PPP (1 μM). Example of a WT MSN stained for internal (blue) and surface (green) GluN2B. Merged channels are displayed to the right of the panel. B) Imaging from MSNs treated with pridopidine (1 μM). Example of a WT MSN stained for internal (red) and surface (green) GluN2B. Merged channels are displayed to the right of the panel. C) Surface/internal GluN2B average of WT and YAC128 MSNs treated with 3-PPP. D) Surface/internal GluN2B average of WT and YAC128 MSNs treated pridopidine. Points represent individual cell averages and error bars represent standard error of the mean. P-values for unpaired t tests are above their respective
bar graphs (WT 3-PPP, n = 27 cells (3 cultures); YAC128 3-PPP, n = 27 cells (3 cultures); WT Pri, n = 22 cells (2 cultures); YAC128 Pri, n = 19 cells (2 cultures).

3.4. Discussion

In this chapter, I studied the effect of S1R and Activin A on normalising GluN2B surface expression, and whether S1R can normalise the action potential-dependent nuclear calcium signalling deficit in YAC128 MSDNs found in chapter 2. To begin I first observed that Activin A is decreased in the culture media of YAC128 cortical-striatal co-cultures versus WT cultures. Activin A is a key component in differentiating human pluripotent stem cells into MSNs (Arber et al., 2015). Specifically, differentiation into MSNs was determined by the expression of DARPP32, a signature marker of MSNs. Indeed, DARPP32 is decreased in striatal lysates of YAC128 mice starting at 6 months of age (Metzler et al., 2010). This could be related to decreased Activin A in mice, and so perhaps exploring whether increased Activin A can increase DARPP32 expression in the striatum of YAC128 could be a compelling future direction. This highlights the potential importance of Activin A in overall MSN health and requires further investigation.

According to my hypothesis, given that S1R agonists have been shown to restore ER calcium levels in HD MSNs (Ryskamp et al., 2017), if ER calcium stores are critical to cytosol to nucleus calcium signalling, then S1R agonists might also be able to: i) normalise the action potential-dependent nuclear calcium signalling deficit observed in chapter 2; and ii) restore the decreased Activin A in media from YAC128 MSNs (Figure 1B). To study this, I imaged MSNs treated with pridopidine and nucleofected with the cytosol-localised RCaMP and nucleus-localised GCaMP. I observed no change in the nucleus to cytosol AUC ratio (GCaMP/RCaMP) after pridopidine treatment. I also treated cultures with either pridopidine or 3-PPP – two S1R agonists
– and measured Activin A in the media. I observed no effect of pridopidine or 3-PPP on Activin A protein levels. These data suggest that S1R agonists are unable to normalise Activin A levels nor the action potential-dependent nuclear calcium signalling deficit in YAC128 cortical-striatal co-cultures. Given the known effect of pridopidine in normalising calcium leak through IP3 receptors (Ryskamp et al., 2017), I initially hypothesised that S1R activation would restore nuclear calcium signalling in YAC128 MSNs. However, the minimal effect of S1R agonists on action potential-dependent nuclear calcium signalling is consistent with the lack of an effect of MCPG or an IP3R1 ASO on action potential-dependent nuclear calcium signalling. Additional pridopidine timepoints and concentrations may need to be tested to strengthen this conclusion. The 1-day treatment was chosen to mimic the same time course for normalisation of IP3R1 previously reported (Ryskamp et al., 2017). The 4-day treatment was also chosen to match the time course for normalisation of homeostatic synaptic plasticity found in our lab (Smith-Dijak et al., 2019). Furthermore, 1 μM pridopidine treatment was used to also mimic the concentration that yielded the largest effect size (Ryskamp et al., 2017; Smith-Dijak et al., 2019). Nevertheless, different combinations of concentrations/timepoints of pridopidine would yield a more thorough exploration of this mechanism.

Activin A has been reported to reduce toxic extrasynaptic NMDA receptor signalling in the hippocampus in a mouse stroke model, as well as decrease phosphorylation of tyrosine residue 1472 on the GluN2B subunit (Lau et al., 2015). Dephosphorylation at this site is known to induce endocytosis of GluN2B-containing NMDA receptors to reduce surface expression (T. Nakazawa et al., 2001). I therefore hypothesised that Activin A would normalise the enhanced surface expression of extrasynaptic GluN2B observed in YAC128 MSNs (Milnerwood et al., 2012). To do this, GluN2B-YFP was overexpressed in MSNs in cortical-striatal co-cultures and stained for
surface and internal GluN2B. Here, measuring the surface to internal ratio of GluN2B fluorescence is a proxy for relative extrasynaptic GluN2B-containing NMDA receptor expression; it has been shown that this increased surface to internal GluN2B ratio observed in YAC128 also showed no synaptic GluN2B change, and this was confirmed with electrophysiological experiments with MK-801 to block synaptic receptors during spontaneous activity followed by whole-cell perfusion of NMDA to record residual extrasynaptic NMDAR-mediated current (Milnerwood et al., 2012). A YFP tagged GluN2B was used because of the lack of reliable N-terminal antibodies (targeting the N-terminal side of GluN2B is essential for surface staining). Indeed, Activin A overexpression was able to reduce surface expression of GluN2B in YAC128 MSNs. The mechanism by which this occurs is presumably through the Activin A receptor ACVR1B; as a future direction, perhaps blocking ACVR1B in the presence of Activin A would elucidate a therapeutic target, and the development of a compound that binds ACVR1B may present a possible avenue for HD treatment.

Interestingly, despite S1R agonists having no effect on action potential-dependent nuclear calcium signalling and Activin A levels in culture media, 3-PPP and pridopidine can decrease GluN2B surface expression selectively in YAC128 MSNs while having no significant impact on WT MSNs. The mechanism by which this occurs seems to be independent of any significant increase in Activin A levels or action potential-dependent nuclear calcium signalling. These data suggest that restoration of ER calcium homeostasis, while not helpful for normalising RCaMP/GCaMP AUC or Activin A levels, has a beneficial effect on normalising GluN2B surface expression. This is an important finding, and encourages further experiments to elucidate the link between NMDAR trafficking and ER calcium. One idea is that ER calcium leak could enhance activation of calcium-dependent protein kinases and/or phosphatases. One such kinase is Casein Kinase 2 (CK2) which phosphorylates Ser1480 of GluN2B thereby uncoupling it from PSD-95.
and promoting movement from synaptic to extrasynaptic sites (Sanz-Clemente et al., 2010, 2013). It has also been shown that enhanced activation of calpain and STEP, which are both calcium dependent, contributed to enhanced extrasynaptic localization of GluN2B-containing NMDA receptors (Gladding et al., 2012). Consistent with this data, calpain activation was found to be enhanced in YAC128 MSNs (Cowan et al., 2008).

Importantly, research in the spinal cord and CA1 pyramidal neurons demonstrate that S1R activation is key in phosphorylating the GluN2B Y1472 residue, which would prevent endocytosis, leading to increased surface GluN2B expression. Furthermore, S1R knockout was shown to prevent GluN2B trafficking to the membrane after spinal cord injury (Castany et al., 2018; Z. Li et al., 2006; Pabba et al., 2014; Salaciak & Pytka, 2022). Moreover, BDNF increased phosphorylation of GluN2B (S. Li et al., 2017), which is consistent with the BDNF-enhancing effect of S1R (Smith-Dijak et al., 2019). Notably, phosphorylation of Y1472 strengthens binding to PSD-95 and keeps GluN2B in the synapse, and dephosphorylation can induce its endocytosis but importantly, can also shift its surface expression to extrasynaptic sites (B. Li et al., 2002; Prybylowski et al., 2005; Won et al., 2016). Therefore, it is possible that S1R agonism is exerting dual effects, to enhance synaptic localization of GluN2B-NMDAR while also reducing calcium-dependent protein kinase and phosphatase activity that promotes the shift to extrasynaptic sites; these effects cannot be distinguished by only using the GluN2B surface/internal assay. Furthermore, PGC-1α, which is important in calcium handling, has its activity reduced HD neurons which leads to mitochondrial dysfunction (Cui et al., 2006). It is possible that S1R agonists could stabilise mitochondrial calcium homeostasis as well, since S1R is highly expressed at the ER-mitochondria associated membranes. To further investigate the possible net shift of surface expression from extrasynaptic to synaptic sites would require repeating the GluN2B
surface/internal assay together with antibody staining for the glutamatergic synaptic marker VGlut1 to determine if its colocalization with GluN2B is increased.
Chapter 4: Activin A AAV restores motor learning of YAC128 mice on a rotarod task to that of WT mice

4.1. Introduction

Activin A is decreased in cortical-striatal co-culture media from YAC128 mice, and Activin A can reduce the surface/internal GluN2B ratio of YAC128 MSNs to that of WT. This change in GluN2B localisation would lead to a rebalance of synaptic vs extrasynaptic NMDA receptors as surface GluN2B serves as a proxy for extrasynaptic NMDA receptors (Milnerwood et al., 2012). With this shift into a higher proportion of synaptic NMDA receptors, neuroprotective signalling pathways would be favoured as synaptic NMDA receptor activation stimulates neuroprotective signalling pathways required for synaptogenesis, synaptic plasticity involved in learning and memory, and activation of pro-survival gene transcription that maintain neuronal health, whereas extrasynaptic NMDA receptor activation opposes plasticity and promotes cell death pathways (Hardingham et al., 2002; Hardingham & Bading, 2010).

Taken together with the evidence that Activin A is neuroprotective (Abdipranoto-Cowley et al., 2009; Arber et al., 2015; Bloise et al., 2019; Kupershmidt et al., 2007; Tretter et al., 2000), I hypothesised that overexpressing Activin A in the striatum of YAC128 mice may ameliorate known motor learning deficits, specifically on the accelerating rotarod task. To study this, I injected Activin A AAV into the dorsal striatum of WT and YAC128 mice at 1.5 months of age. I then tested their motor learning ability in the rotarod task as the YAC128 model suffers motor learning deficits, as evidenced by slower learning of the rotarod task as early as 4 months of age (Brooks et al., 2012; Garcia-Miralles et al., 2017).
4.2. Methods

4.2.1. Animals

A colony of FVB/N WT and heterozygous YAC128 (line 53) mice (Slow et al., 2003) were bred and maintained in the University of British Columbia Preclinical Discovery Centre according to guidelines of the Canadian Council on Animal Care, under the approved protocols A17-0295 and A19-0076.

4.2.2. Animal surgeries; AAV injections

To test the therapeutic effects of Activin A, mice were injected with AAVs into the dorsal striatum bilaterally at 1.5 months of age. Lidocaine (50 μL) was injected subcutaneously at the surgical site. During surgery, mice were anaesthetised with isoflurane (4% initially, then maintained at 1.5%) and given buprenorphine (0.05 mg/kg), meloxicam (5 mg/kg at 1 mL/kg) both via subcutaneous injection for analgesia. Ophthalmic ointment was applied to both eyes after induction of anaesthesia. The mouse’s head was shaved, then ethanol and betadine was applied to disinfect the incision site. A 0.5 cm medial incision (anterior-posterior) was made to gain access to the skull. Holes in the skull were drilled using a dental drill at the coordinates (relative to the bregma) 2.25 mm lateral, and 0.75 mm rostral. A stereotaxic injector (Fofia ZS006) was used to inject 1 μL (5 x 10⁹ viral count/L of the AAV in both hemispheres at the previous coordinates and at a depth of 3.00 mm. A manipulator (Sutter Instruments) was used to accurately measure these distances. AAVs were injected at a rate of 2 nL/s, and the injector was not moved from the injection site for 10 minutes to allow passive diffusion of the AAV. The injector was then moved to the contralateral site and the same injection parameters were used. AAVs injected were either an Activin A AAV (rAAV-hSyn-inhba-HA/hSyn-tdimer, gift from H. Bading, Heidelberg U) at a
concentration of $5 \times 10^9$ viral count/mL, or a control mCherry AAV (rAAV-CMV/CBA- mCherry NLSr, gift from H. Bading, Heidelberg U) at a concentration of $5 \times 10^9$ viral count/mL. The incision site was then sutured, and the mouse was given time to recover before being returned to its cage. Mice were then monitored daily for 5 days to ensure proper recovery.

4.2.3. Rotarod

To study motor learning and coordination, mice were assessed using the accelerating rotarod task at 6 months of age. The rotarod is a spinning rod that mice must run atop of to prevent themselves from falling. The rod accelerates from 5 to 40 RPM over the course of 300 seconds. Latency to fall was recorded. A fall also included the mouse holding on to the rod and performing a complete rotation. If the mouse stayed on the rod for 300 seconds, the trial ended and the latency to fall was noted as 300 seconds. For each mouse, 3 trials separated each by 2 hours were done each day over 4 consecutive days. Day averages of latency to fall off the rotarod were calculated by averaging that of the 3 trials within a day.

4.2.4. Slice preparation

Mice were anaesthetised with isoflurane and decapitated. The brain was quickly removed and the hemispheres were separated by bisecting along the midline. Sagittal slices of 250 µm thickness of each hemisphere were sliced using a vibratome (Leica) in ice-cold aCSF. Each slice was then transferred to aCSF at 37°C for 30 minutes, and then 30 more minutes at room temperature before electrophysiological experiments. The ice cold aCSF used for slicing contained (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 2.5 MgCl$_2$, and 10 glucose. All other aCSF contained (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2 CaCl$_2$, 1 MgCl$_2$, and 10 glucose. The aCSF had a pH of 7.3-7.4 and an osmolarity of 310 Osm/L. aCSF was
continuously oxygenated with carbogen (95% O₂/5% CO₂) during slicing, recovery, and all experiments. Once transferred to the recording chamber, slices were continuously perfused with room temperature aCSF containing picrotoxin (50 µM; Tocris Bioscience) to block GABAA receptors.

### 4.2.5. Slice fixing and mounting

To ensure that slices were expressing the injected plasmids in the striatum, brain slices were fixed in 4% paraformaldehyde (PFA) and 2% sucrose for 30 minutes at room temperature. Slices were then mounted onto slides using fluoromount G (Southern Biotech). Mounted slides were then imaged with the Zeiss Axio Observer Z1, using a 20x objective.

### 4.2.6. Electrophysiology

Intracellular whole-cell electrophysiological recordings were done using the whole-cell patch clamp technique in voltage-clamp mode. Recordings were acquired with an amplifier (MultiClamp 700A Molecular Devices) and a digitizer, digitised at 20 kHz and acquired with a low pass 1 kHz filter. Pipettes for electrodes were pulled from borosilicate glass capillaries using a micropipette puller (Narishige International for the recording electrode and stimulating electrode). The intracellular solution was cesium-based – cesium blocks potassium channels and allows for a better voltage-clamp – and contained the following in mM: 130 cesium methanesulfonate, 5 CsCl, 4 NaCl, 1 MgCl₂, 5 EGTA, 10 HEPES, 5 QX-314 chloride, 5 MgATP, 0.5 MgGTP and 10 sodium phosphocreatine. The intracellular solution had a pH of 7.2-7.3 and an osmolarity of 290 (±3) mOsm/L. Only MSNs with an access resistance < 17 MΩ were included in the analysis. For all cells, recording only began after 5 minutes of acquiring access to allow the
electrode solution to equilibrate with the intracellular space. To record spontaneous excitatory postsynaptic currents (sEPSCs), cells were voltage-clamped at -70 mV.

**4.2.7. TBOA experiment**

To study extrasynaptic NMDA receptors, a whole-cell voltage clamp was achieved, and trains of stimulation (20 Hz for 500 ms) were generated with a stimulating electrode placed ~200 µm dorsal to the recorded cell. The cell was voltage clamped at +40 mV to relieve Mg$^{2+}$ block from NMDA receptors, and a 50 ms voltage step to -80 mV was applied every 30 s to monitor the access resistance. CNQX (10 µM; AMPA receptor antagonist) and glycine (10 µM; NMDA receptor coagonist) were used to isolate NMDA receptor current. DL-TBOA (10 µM) – a glutamate transport inhibitor – was used to elicit glutamate spillover beyond the synapse into extrasynaptic sites to activate extrasynaptic NMDA receptors. Analysis of electrophysiology data was performed using Clampfit 10.7 (Molecular Devices).

**4.2.8. Data analysis and statistics**

All statistical analysis was done using Graphpad Prism 9. For analysis of the rotarod data, a two-way ANOVA was done to explore the effect of Activin A, the genotype, and the interaction between Activin A and genotype on latency to fall off the rotarod.

For the TBOA experiment, from the trains of stimulation (20 Hz for 500 ms), a ratio (10/1) of the peak amplitude of the final postsynaptic event (from the 10th stimulation) over the peak amplitude of the first postsynaptic event (from the first stimulation) was computed. The trains of stimulation combined with the DL-TBOA application promotes extrasynaptic NMDA receptor activation; the first event in the train is largely synaptic whereas the last event has more extrasynaptic NMDA receptor contribution. The 10/1 ratio gives a measure of the proportion of
extrasynaptic NMDA receptor contribution relative to synaptic NMDA receptor. Furthermore, I measured the decay time divided by the peak amplitude of the postsynaptic event from the 10th stimulation. This would give a relative measure of the NMDA receptor subtype present as GluN2B-containing NMDA receptors have longer decay times than GluN2A-containing NMDA receptors.

For analysis of sEPSCs, events were detected with a template search, and only events with an amplitude greater than 8 pA were counted in the analysis. Cell averages of amplitudes and frequency of events were computed and a two-way ANOVA was done to explore the effect of Activin A, the genotype, and the interaction between Activin A and genotype on MSN spontaneous activity. Furthermore, statistics such as cell capacitance, membrane resistance, and the tau of a 10 mV step (from -70 mV to -60 mV) were measured and also analysed using a two-way ANOVA.

4.3. Results

4.3.1. Activin A increases the ability of YAC128 mice to learn the rotarod task

To test whether Activin A can improve motor learning, I injected 1.5 month old mice with either an Activin A AAV or a control AAV as per section 4.2.2. They were then tested at 6 months of age using the rotarod as per section 4.2.3. Firstly, Figure 4.1A shows clear expression of the Activin A AAV; in this image, the right and left hemispheres were injected with 1 μL and 0.5 μL of the AAV, respectively, to test the AAV. 1 μL was used for subsequent experiments because it offered more coverage of the striatum. On the rotarod, YAC128 mice injected with the control AAV performed significantly worse than WT injected with control AAV. In contrast, YAC128 mice injected with Activin A AAV performed almost as well as WT mice (Figure 4.1B-C). Also
of note, the Activin A had no effect on the performance of WT mice (Figure 4.1B-C). Furthermore, this result was not dependent on the mouse’s weight, as the Activin A did not have an effect on the well-known increased weight of YAC128 mice compared to WT mice (Figure 4.1D) (Pouladi et al., 2010; Van Raamsdonk et al., 2006).
Figure 4.1: Activin A increases the ability of YAC128 mice to learn the rotarod task. A) Example of a coronal brain slice expressing Activin A (right hemisphere injected with 1 μL AAV and left hemisphere injected with 0.5 μL AAV). B) Latency to fall day averages of WT and YAC128 male and female mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. C) Top: Latency to fall trial averages of WT and YAC128 male and female mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. Bottom: Latency to fall day averages of individual WT and YAC128 male and female mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. D) Average weights of WT and YAC128 male and female mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. E) Left: Latency to fall trial averages of WT and YAC128 female mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. Right: Latency to fall day averages of WT and YAC128 female mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. F) Left: Latency to fall trial averages of WT and YAC128 male mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. Right: Latency to fall day averages of WT and YAC128 male mice at 6 months of age treated either with Activin A AAV or a control mCherry AAV. Points represent individual mice. Error bars represent standard error of the mean. P-values for two-way ANOVA test are above the bar graph (the p values in panel B are for day 4) (For B: WT + mCherry, n = 11 mice; WT + Activin A, n = 12 mice; YAC128 + mCherry, n = 9 mice; YAC128 + Activin A, n = 11 mice; Bonferroni multiple comparisons test in panel B: mCherry vs. Activin A: WT p-value = >0.9999; YAC128 p-value = <0.0001) (For E: WT + mCherry, n = 0 mice; WT + Activin A, n = 6 mice; YAC128 + mCherry, n = 5 mice; YAC128 + Activin A, n = 3 mice; there was no WT + mCherry group so a two-way ANOVA could not be done; more samples will be collected) (For F: WT + mCherry, n = 11 mice; WT + Activin A, n = 6 mice; YAC128 + mCherry, n = 4 mice; YAC128 + Activin A, n = 8 mice; Bonferroni multiple comparisons test in panel B: mCherry vs. Activin A: WT p-value = >0.9999; YAC128 p-value = <0.0001).
4.3.2. Activin A has no apparent effect on extrasynaptic NMDA receptor currents

To study the effect of Activin A on extrasynaptic NMDA receptors, I recorded from whole-cell patch voltage-clamped MSNs, measuring the current response to train stimulation, as described in section 4.2.7. The brain slice recordings were carried out within 2 months of the mice completing the rotarod task. The pilot data from only 2-3 mice in each group suggest that both decay time/peak (Figure 4.2E) and the 10/1 peak ratio (Figure 4.2F) are significantly smaller in WT MSNs than the YAC128 MSNs, and that there is no significant Activin A effect on either decay/peak (Figure 4.2E) nor the 10/1 peak ratio (Figure 4.2F). Further experiments will be done to increase the number of mice recorded from each group.
Figure 4.2: Activin A has no effect on extrasynaptic NMDA receptor currents. A) Example of a trace showing a stimulation train (20 Hz for 500 ms) during DL-TBOA in a WT + mCherry MSN. B) Example of a trace showing a stimulation train (20 Hz for 500 ms) during DL-TBOA in a YAC128 + mCherry MSN. C) Example of a trace showing a stimulation train (20 Hz for 500 ms) during DL-TBOA in a WT + Activin A MSN. D) Example of a trace showing a stimulation train (20 Hz for 500 ms) during DL-TBOA in a YAC128 + Activin A MSN. E) Time course of decay/peak averages (every 30 seconds) of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. F) Time course of decay/peak averages (every 30 seconds) of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. Error bars are standard errors of the mean. (WT + mCherry, n = 3 cells (3 mice); WT + Activin A, n =
2 cells (2 mice); YAC128 + mCherry, n = 4 cells (3 mice); YAC128 + Activin A, n = 4 cells (3 mice)).

4.3.3. Effect of Activin A on cell health

To assess cell health, sEPSCs amplitude and frequency averages as well as cell capacitance, membrane resistance, and the tau of a 10 mV step (from -70 mV to -60 mV) were measured and also analysed using a two-way ANOVA. sEPSCs were acquired as per section 4.2.6. The pilot data from 2-3 mice in each group show no significant genotype or Activin A AAV effects on any of these parameters (Figure 4.3B-F). Further experiments will be done to increase the number of mice recorded from each group.
Figure 4.3: Effect of Activin A on cell health. A) Example of a trace showing sEPSCs in a WT + mCherry MSN. B) sEPSCs amplitude averages of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. C) sEPSCs frequency averages of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. D) Cell capacitance averages of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. E) Cell membrane resistance averages of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. F) Tau averages of a 10 mV step (from -70 mV to -60 mV) of WT and YAC128 MSNs previously treated with either Activin A AAV or mCherry AAV. Error bars represent standard errors of the mean. Points represent individual cells. (WT + mCherry, n = 5 cells (3 mice); WT + Activin A, n = 2 cells (2 mice); YAC128 + mCherry, n = 3 cells (3 mice); YAC128 + Activin A, n = 5 cells (3 mice).
4.4 Discussion

With the compelling evidence that Activin A is decreased and that its overexpression leads to the normalisation of GluN2B surface expression in YAC128 MSNs co-cultured with cortical neurons, I sought to test its therapeutic effectiveness in ameliorating YAC128 mice performance on a motor learning task. To do this I used the accelerating rotarod task as YAC128 mice suffer motor learning deficits as evidenced by a slower learning of the rotarod task as early as 4 months of age (Brooks et al., 2012; Garcia-Miralles et al., 2017). Given the progressive nature of the disease, I injected mice with the Activin A AAV at an early time point (1.5 months of age) in the disease process. For example, daily pridopidine treatment in YAC128 mice starting at 2 months of age led to improved performance on the rotarod task compared to vehicle treatment (Garcia-Miralles et al., 2017).

On the rotarod, Activin A AAV treated YAC128 male mice performed significantly better than control AAV treated YAC128 mice, and almost as well as WT male mice. As for the female YAC128 mice, they also seemed to perform better with Activin A treatment, although numbers were low and need to be increased. Moreover, there seems to be a sex difference between the male and female YAC128 mice; male YAC128 mice treated with mCherry AAV perform worse than their female counterparts, and the Activin A AAV also seems to have a larger effect on rotarod learning in males vs females. More animals are needed to verify this conclusion. Activin A AAV treatment also does not seem to negatively impact WT mice performance, suggesting that Activin A has low toxicity. Furthermore, the effect of Activin A on the rotarod task was independent of weight as Activin A had no effect on that metric.

A sex difference is unsurprising as recent work suggest some sex differences in progression
of disease manifestations in both human gene mutation carriers and in rodent models of HD. These studies indicate that among HD patients, women have more severe motor symptoms and that with men, loss of functional abilities was more correlated with cognitive deficits (Zielonka et al., 2018). In rodents however, males exhibit motor incoordination earlier and more severely than females (Cao et al., 2018; Zarringhalam et al., 2012), along with stronger effects of HD gene expression on circadian rhythm (Kuljis et al., 2016) and earlier changes in corticostriatal circuit function and striatal neurochemistry in males (Padovan-Neto et al., 2019). In rodents, hormonal factors account for some of these sex differences (Bode et al., 2008; Hsu et al., 2011). This may also play a role in HD patients, suggesting that disease progression in pre- vs. post-menopausal women is an important area of future investigation.

Another study examined the effect of early pridopidine treatment on rotarod performance of YAC128 mice. In this study, both male and female mice were treated with pridopidine daily by oral gavage 5 days/week starting at 1.5 months of age. The mice were tested every 2 months starting at 2 months of age on the accelerating rotarod (Garcia-Miralles et al., 2017). When comparing the 6 month data, the 30 mg/kg treatment ameliorated the YAC128 mice performance on the rotarod task, but not to the same extent as the Activin A treatment although the study does not report a statistical test for comparing performance on the rotaord between 6 month old WT mice and YAC128 mice treated with 30 mg/kg pridopidine. Finally, tetrabenazine also ameliorates the performance of YAC128 mice on the rotarod but only after 9 months of age; there was no effect at 6 months of age on the rotarod task (Wang et al., 2010). This positions Activin A as an exciting therapeutic target for HD.

Given the effect of Activin A to normalise GluN2B surface expression in YAC128, I explored whether Activin A could decrease extrasynaptic NMDA receptor currents using whole-
cell voltage-clamp. Mice that had completed the rotarod task were euthanized, and their brains were sliced for electrophysiological recordings. With the low numbers obtained so far, no significant effect of Activin A on extrasynaptic NMDA receptors was observed, suggesting that the beneficial effects of Activin A may be independent of extrasynaptic NMDA receptors. It has been reported that cortical-striatal connectivity and MSN dendritic branching are reduced in HD (Cepeda & Levine, 2022; Raymond et al., 2011; Veldman & William Yang, 2018). Indeed, YAC128 MSNs in cortical-striatal co-cultures exhibit decreased dendritic arborisation points than their WT counterparts (Schmidt et al., 2018). Given that Activin A has been shown to increase spine density in hippocampal neurons (Hasegawa et al., 2014; Shoji-Kasai et al., 2007), it would be interesting to study the effect of Activin A on dendritic morphology in cortical-striatal co-cultures and brain slices as this could be another possible mechanism that could explain the improved rotarod performance of YAC128 mice after Activin A AAV treatment. Furthermore, as mentioned in Chapter 3, Activin A is a key component in differentiating human pluripotent stem cells into MSNs (Arber et al., 2015). Specifically, differentiation into MSNs was determined by the expression of DARPP32, a signature marker of mature MSNs. Indeed, DARPP32 is decreased in striatal lysates of YAC128 mice starting at 6 months of age (Metzler et al., 2010). This could be related to decreased Activin A in mice, and so perhaps exploring whether increased Activin A can increase DARPP32 expression in the striatum of YAC128 could be a compelling future direction. These proposed mechanisms highlights the potential importance of Activin A in overall MSN health and requires further investigation.

Another possible mechanism that could explain increased rotarod performance is the reported ability of Activin A to reduce ER stress-induced apoptosis and autophagy of PC12 cells—a cell line derived from a transplantable rat pheochromocytoma (Xue et al., 2017). Activin A
was able to inhibit the expression of proteins related to apoptosis such as cleaved-caspase-3, cleaved-caspase-12, and C/EBP homologous protein (CHOP). ER stress associated proteins – such as inositol requiring enzyme-1 (IRE1), tumour necrosis factor receptor-associated factor 2 (TRAF2), apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK) and p38 – were also downregulated by Activin A (Xue et al., 2017). Given that calcium handling is impaired in HD, and that calcium is a key regulator of ER stress (Bahar et al., 2016), studying this pathway may elucidate a mechanism for Activin A’s effect on improved motor learning performance in YAC128 mice.
Chapter 5: Conclusions

5.1. Summary of findings

In this thesis I explored the mechanistic underpinnings of HD and demonstrated nuclear calcium signalling deficits while also discovering a new therapeutic avenue. Using cortical-striatal co-cultures nucleofected with a cytosol-localised RCaMP and a nucleus-localised GCaMP, I observed that the ratio of the AUC (GCaMP/RCaMP) was decreased in YAC128 mice, indicating a deficit in transmission of cytosolic calcium signals to the nucleus; however, I could not successfully isolate the cause of this genotype difference. In addition, I found that Activin A, whose expression is nuclear calcium-dependent and can downregulate extrasynaptic NMDARs (Lau et al 2015), was decreased in YAC128 cortical-striatal co-cultures. Although S1R agonists have been shown to restore ER calcium homeostasis in HD striatal neurons (Ryskamp et al., 2017), the reduction in Activin A was not normalised by S1R agonists, which also failed to correct the cytosol to nucleus calcium signal transmission deficit. However, both Activin A and S1R agonists were able to decrease the surface/internal GluN2B ratio, a proxy for extrasynaptic NMDARs, in striatal neurons from YAC128 but not WT cortical-striatal cultures. Finally, overexpression of Activin A in striatum was effective in normalising the performance of YAC128 mice on the rotarod task.

To elaborate on the calcium imaging findings in striatal neurons, I found that high dose ryanodine (RyR antagonist), but not MCPG (mGluR1/5 antagonist that should lower intracellular IP3 levels) nor an IP3R1 ASO, significantly reduced cytosolic and nuclear calcium signals proportionately; RyRs but not IP3 receptors therefore contribute significantly to nuclear and cytosolic spontaneous action potential-dependent calcium signals. Furthermore, nifedipine
application significantly decreased cytosolic, but not nuclear calcium signals, placing the role of L-type CaV channels as being more involved in cytosolic calcium signals vs nuclear calcium signals. Finally, D-APV completely abolished cytosolic and nuclear calcium signals, suggesting an essential role for NMDA receptors as the initiator of action potential-dependent calcium in striatal neurons.

Given the nuclear calcium signalling deficit observed in YAC128 cortical-striatal co-cultures, I studied whether Activin A was decreased. An Activin A ELISA revealed that Activin A levels were indeed lower in YAC128 culture media versus WT. Furthermore, overexpression of Activin A in striatal neurons was able to decrease the aberrant surface/internal GluN2B ratio observed in YAC128 co-cultures, assessed via GluN2B-YFP expression and surface/internal immunocytochemistry. Contrary to my hypothesis, S1R agonists were not able to normalise decreased Activin A, nor normalise the decreased AUC ratio (GCaMP/RCaMP) observed in YAC128 co-cultures. However, S1R agonists were able to decrease the surface/internal GluN2B ratio more in YAC128 versus WT co-cultures.

Finally, I injected an Activin A AAV into mouse striatum bilaterally to test the effect of Activin A overexpression on the rotarod task. Mice were injected at 1.5 months of age, and were tested on the rotarod at 6 months of age when YAC128 mice performed significantly worse on the task versus WT mice. Activin A AAV treatment was able to improve the learning of the task for YAC128 mice to match that of WT mice as seen by the increase in latency to fall through 4 days of trials. Furthermore, Activin A had no effect on the rotarod performance of WT mice. Electrophysiology data revealed that this behavioural change is not correlated with a decrease in extrasynaptic NMDA receptor current in YAC128 MSNs in acute slice terminal experiments.
5.2. Overall discussion

My data is consistent with some features of my hypothesis, but not others. Calcium signalling to the nucleus is indeed decreased in YAC128, but the mechanism underlying this decrease could not be identified in my experiments, which probed a role for differences in ER calcium release, NMDAR-mediated calcium influx and L-type calcium channel involvement. However, I uncovered some mechanistic underpinnings of nuclear calcium signalling in MSNs that are novel. Most research regarding the mechanism of nuclear calcium signalling has been done in the hippocampus or cortex, and usually with excitatory neurons (Bading, 2013; Bengtson & Bading, 2012; Deisseroth et al., 1998; Weislogel et al., 2013; Westenbroek et al., 1990). In these cells, RyR is known to be involved with nuclear calcium signalling, and IP3 receptors have minimal impact on nuclear calcium signalling (Berridge, 2016; Taylor et al., 2004). NMDA receptors are also reported to be important in nuclear calcium signalling (Bading, 2013; Bengtson & Bading, 2012; Karpova et al., 2012), but not to the degree that I observed where D-APV completely abolished both cytosolic and nuclear calcium signalling. Furthermore, calcium flux through L-type CaV channels has been reported to have preferential access to the nucleus (Bading, 2013; Westenbroek et al., 1990), whereas my data shows no significant change in nuclear calcium response after nifedipine application even though AP-induced cytosolic calcium transients were significantly reduced. One difference between my data and that of the literature is that I was observing spontaneous action potential-dependent calcium signals whereas other papers induce trains of action potentials. Furthermore, calcium influx through L-type CaV channels can bind calmodulin-dependent protein kinase II (CaMKII) in the cytosol which then translocates to the nucleus to modulate transcription, bypassing the need for free calcium entry to the nucleus (Perfitt et al., 2020). Calcium from L-type CaV channels may therefore have preferential access to the
nucleus only after a train of stimulation. This can and should be tested in striatal neurons in future experiments.

Activin A was decreased in YAC128 co-culture media. I hypothesised that the decrease in Activin A was due to decreased calcium signalling to the nucleus. Though I found decreased nuclear calcium signalling and decreased Activin A, I do not have results showing a cause-and-effect relationship. However, the transcription of the monomer of Activin A, inhibin beta a, is controlled by the cAMP response element-binding protein (CREB), whose activity level is regulated by nuclear calcium (Ardekani et al., 1998). CREB activity is increased by phosphorylation at Ser133 by protein kinase A (PKA), CaMKII and IV, MAPK, and other kinases (Alberini, 2009; Wang et al., 2018). Phosphorylated (activated) CREB is also shown to be reduced in the striatum of YAC128 mice as early as one month of age (Milnerwood et al., 2010). This decrease in nuclear phospho-CREB is mainly a downstream consequence of enhanced extrasynaptic GluN2B-containing NMDA receptor signalling, and blockade of these receptors with low dose memantine (which preferentially blocks extrasynaptic NMDA receptors) restores CREB activation (Dau et al., 2014; Milnerwood et al., 2010). It would be interesting to see whether memantine treatment could normalise Activin A protein expression in YAC128 cortical-striatal co-culture media. Notably, memantine has also been shown to bind S1Rs (Peeters et al., 2004). However, given that neither 3-PPP nor pridopidine could normalise Activin A in YAC128 co-culture media, memantine’s possible effect on Activin A expression would most likely be due to extrasynaptic NMDA receptor blockade. It is also possible that the lack of an effect with S1R agonists on Activin A or nuclear calcium signalling may be due to the concentration or the time course of the treatment, which could be further explored in future experiments.
Though Activin A AAV treatment improved the performance of YAC128 mice in learning the rotarod task, this was seemingly not due to a decrease in extrasynaptic NMDA receptors (though a larger sample size for the electrophysiology data is needed to confirm this). It is interesting that Activin A is reported to induce DARPP32 expression in human pluripotent neuronal precursor cells, thereby contributing to their differentiation into striatal neurons (Arber et al., 2015). A key marker of MSN “identity”, DARPP32 mRNA shows early down-regulation in HD mouse models (Bibb et al., 2000) and its protein expression is decreased in YAC128 mice beginning at 6 months of age (Metzler et al., 2010). Therefore, assessing the expression of DARPP32 in striatal tissue would provide important data on the general health of the MSN population; if Activin A could elevate DARPP32 in YAC128 striatum, this could help explain the observed behavioural improvement in YAC128 mice. However, it would not offer a mechanistic explanation for the effect of Activin A. One other possible explanation for the effect of Activin A on the rotarod performance is its ability to reduce ER stress-induced apoptosis (Xue et al., 2017). Indeed, apoptotic pathways such as c-Jun N-terminal kinase (JNK) and p38 Mitogen-activated Protein Kinase (MAPK) – that are increased in YAC128 striatum (J. Fan et al., 2012) – are reduced after Activin A treatment in PC12 cells (Xue et al., 2017). It would thus be interesting to look at the ratio of phosphorylated to non-phosphorylated p38 and c-Jun respectively after Activin A treatment in YAC128 striatum to assess the level of activation of the p38 MAPK and JNK pathway.

5.3. Limitations

A clear limitation of this work is that the variability in cortical-striatal co-cultures made it difficult to detect genotype differences using the RCaMP-GCaMP imaging paradigm. Due to this
variability, a large sample size was needed to detect a difference. Furthermore, drug treatments could not elucidate a mechanistic difference to explain the decreased nuclear calcium signalling observed in YAC128 cultures; it is therefore difficult to distinguish whether the lack of a difference is due to the presence of no genotype difference or the experiment being underpowered. One issue is that the transfection rate of nucleofections is low (Viesselmann et al., 2011). A technique with a higher transfection rate would enable capturing more cells in a field of view, thus increasing the rate of data acquisition and the sample size. Another possibility is that these cultures were imaged around DIV17-19. Perhaps if MSNs were imaged at a later time point beyond DIV19, a greater genotype difference may have been observed.

Though I imaged the cytosol and nucleus compartments simultaneously, it does not paint the whole picture. ER calcium is important for proper protein folding and a lack of calcium leads to misfolded protein. Though the presence of hypersensitive IP3 receptors as well as the presence of leaky RyRs has led to the hypothesis that ER calcium stores are depleted in YAC128 MSNs, ER calcium of YAC128 MSNs has never directly been measured. First, calcium store depletion would lead to store-operated calcium entry, a mechanism to rapidly refill calcium stores. Store-operated calcium entry is enhanced in YAC128 MSNs (Vigont et al., 2015; J. Wu et al., 2011). Taken together, it is difficult to predict whether ER calcium stores are depleted or not, as there are two competing mechanisms in the form of enhanced calcium efflux through IP3 receptors and RYRs, and increased calcium influx through the store-operated response. Altogether, there seems to be more calcium movement across the ER membrane in YAC128 MSNs, and capturing this with the help of a ratiometric calcium indicator would better elucidate the difference between WT and YAC128 MSNs in calcium handling.
Another limitation in my cortical-striatal co-culture data is that it only accounts for cortical and striatal neurons. Though this allows to isolate the cortical-striatal synapse, this is not necessarily the case in a live brain where astrocytes and microglia also play a role in cellular function. For example, astrocytes are important in glutamate reuptake, important in managing the duration of glutamate in the synapse, and also important in preventing glutamate spillover to extrasynaptic sites. Some of the experiments should be done in brain slices or in vivo, such as imaging calcium simultaneously in cytosolic and nuclear compartments, to validate the in vitro data from cortical-striatal co-cultures.

5.4. Significance and Future Directions

In this thesis, I have uncovered some new mechanistic underpinnings of HD. First, I found that nuclear calcium signalling is impaired in YAC128 MSNs, and I have elucidated the role of various calcium channels in synapse-to-nucleus signalling in MSNs. Second, I have shown that Activin A protein expression is decreased in YAC128 cultures, and that its overexpression can normalise GluN2B surface expression. Importantly, Activin A overexpression in the striatum was able to improve learning of the rotarod task for YAC128 mice to match that of WT mice. Firstly, there may be a sex difference whereby male YAC128 mice treated with mCherry AAV perform worse than their female counterparts, and the Activin A AAV also seems to have a larger effect on rotarod learning in males vs females. More animals would be needed to verify this conclusion. Furthermore, the effect of Activin A on rotarod performance of YAC128 mice is of significance as no other treatment in the literature has had this same magnitude of effect on the rotarod task. Given this, the possible mechanism of Activin A should be explored as it may elucidate other
therapeutic targets. To begin, no agonist of ACVR1B other than recombinant Activin A is available; perhaps the development or search for such a compound would be beneficial as a treatment for HD. Alternatively, exploring the pathway downstream of the ACVR1B – the Smad intracellular protein signalling cascade (Namwanje & Brown, 2016) – and the effect of proteins that halt that pathway such as follistatin (Leung & Peng, 2003) may also shed some light on the beneficial effects of Activin A on YAC128 MSNs. This would also be important since the mechanism linking ACVR1B to its effects on NMDA receptors, seen previously (Lau et al., 2015) and also in this thesis, remains to be understood.

Together with Activin A, an approach to normalise nuclear calcium signalling must also be discovered and subsequently tested in YAC128 mice to explore whether Activin A is normalised in YAC128, and whether the therapeutic effects seen from Activin A in the rotarod task on YAC128 mice can be recapitulated. Perhaps the RCaMP-GCaMP paradigm at a later time point than DIV19 in co-cultures would increase the genotype difference, making studying a way to normalise nuclear calcium signalling in YAC128 MSNs more feasible.

Finally, the effect of pridopidine and 3-PPP on decreasing GluN2B surface expression in YAC128 MSNs cannot be ignored. Taken together with their inability to normalise Activin A expression, though at the same time having a similar effect on the performance of YAC128 mice on the rotarod (Garcia-Miralles et al., 2017), it seems that S1R agonists play a similar role to Activin A, while doing so through a different mechanism. This presents the possibility of a conjunctive therapy of an ACVR1B agonist combined with pridopidine, which may have additive effects in HD treatment. This would require further testing in animal models of HD, and may be beneficial to the treatment of HD in the future.
Altogether, my thesis presents a compelling therapeutic avenue for HD. This is the beginning of the work needed to fully understand the mechanism of Activin A in HD, and the mechanisms by which to normalise Activin A expression. The body of work exploring the effects of Activin A in the striatum is still in its early days, but elucidating this mechanism would add another therapeutic approach to the arsenal in the search for an effective treatment for HD, a disease that currently still has no disease-modifying treatment available.
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Appendix

Dendritic segmentation code:

```python
from tifffile import imread
from aicsimageio.readers import CziReader
from skimage.filters import threshold_otsu
from skimage.exposure import adjust_gamma, is_low_contrast
import h5py
import matplotlib.pyplot as plt
import matplotlib.image as mpimg
import napari
import numpy as np
import numpy.ma as ma
import subprocess
import os, sys

%gui qt

In []:

# Path to CZI File
FILE_PATH = "mnt/5404b8a5-71b7-4464-9a1e-b40cd26fac58/Data_Drive/Wissam/Dendrite_Movies/Cell_351-WT-Green_Channel_Just_ECF_at_0_minutes_DIV19.czi"
FILE_PATH = '/home/peter/Downloads/Cell 368-WT.tif'

# Cell's ID
CELL_ID = 'Cell_368-WT'

# Path to ilastik launch script
ILASTIK_LOC = '/home/peter/Applications/ilastik-1.3.3post3-Linux/run_ilastik.sh'

# Path to ilastik classifier project
ILASTIK_PRO = 'neuron_classifier.ilp'

SEG_DIC = {0:"Background",
           1:"Soma",
           2:"Dendrites"
}
HZ = 10

if FILE_PATH[-3:] == 'czi':
    print('CZI File Format')
    Czi_File = CziReader(FILE_PATH)
    movie = Czi_File.data[0,:,0,:,0]
    del Czi_File

if FILE_PATH[-3:] == ('tif' or 'iff'):
    print('TIF File Format')
    movie = imread(FILE_PATH)

max_project = np.max(movie, axis=0)
```

In []:

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In []:

In []:

In []:

In []:

In []:
brighter = adjust_gamma(max_project, gamma=.25)
OUTPUT = '%s_Brighter.npy' % (CELL_ID)
np.save(OUTPUT, brighter/brighter.max())

launch_args = [ILASTIK_LOC,
               '--headless',
               '--project='+ILASTIK_PRO,
               '--export_source=probabilities',
               OUTPUT]
subprocess.run(launch_args)

pixel_classifier = h5py.File('%s_Brighter_Probabilities.h5' % (CELL_ID))
classes = pixel_classifier['exported_data']

labels = np.zeros(max_project.shape)
label = 0

for i in range(classes.shape[2]):
    class_mask = classes[:,:,i].copy()
    class_mask[class_mask<.75]=0
    test= max_project*class_mask
    if i is not 0:
        test = brighter * class_mask
        threshold =threshold_otsu(test)
        print(threshold)
        test[test<threshold]=0
        test[test>threshold]=1
        labels[test>0]=label
    label+=1
plt.imshow(test)
plt.show()

# Generates ROIs
# 0 - Background
# 1 - Soma
# 2 - Dendrites
labels = np.zeros(max_project.shape)
label = 0
for i in range(classes.shape[2]):
    print(i)
    labels[classes[:,:,i]>,.60]=label
    label+=1
plt.imshow(labels)

# Clean up stuff from memory
def test
def classes
def pixel_classifier
def

# Labels can be edited in the Napari window
viewer = napari.view_image(movie, name='movie')
viewer.add_labels(labels, name='Segments')
```python
if os.path.exists('Output_Traces') == False:
    os.makedirs('Output_Traces')

for i in np.unique(labels):
    if i != 0:
        trace_movie = movie.copy()
        trace_movie = np.broadcast_to(labels==i, trace_movie.shape)
        trace_movie.setflags(write=1)
        trace_movie[trace_movie != 0] = 0
        trace_movie = movie * trace_movie

        mask_arr = trace_movie == 0
        mx = ma.masked_array(trace_movie, mask=mask_arr)
        mean = np.mean(axis=(1,2))
        print("Ca2+ Trace for the %s" % SEG_DIC[i])
        plt.plot(mean)
        plt.show()

        np.savetxt("Output_Traces/{0}_{1}.csv".format(CELL_ID, SEG_DIC[i]),
                   mean, delimiter="", )
        del trace_movie
        del mask_arr
        del mx
```

```
import natsort
import math
import matplotlib.pyplot as plt
import numpy as np
import os
import pandas as pd
import scipy.signal as signal
import skimage.filters as filters
from IPython.display import display, clear_output
from ipywidgets import interact, interactive, fixed, interact_manual
import ipywidgets as widgets
from PIL import Image

# The Folder containing sub-directories to include in analysis
DATA_DIRECTORY = "Output_Traces"
# Frequency frames were collected
```
HZ = 10

""
""
""
""
""
""
# To install pyneurotrace use this:
# pip install --upgrade "git+https://github.com/padster/pyNeuroTrace#egg=pyneurotrace&subdirectory=pyneurotrace"

Out[24]:
'
'

# Change output figure size
# ...needs to be in its own cell for some reason...
plt.rcParams['figure.figsize'] = [20, 5]

In [25]:

""
Performs fast nonnegative deconvolution on pmt signal to solve for minimum MSE photon rate

trace : The data to be deconvolved
tau : The time constant of the PMT, in data samples
return : estimated photon rate

A matlab version is also available on request.
For details on how this works, see:
""
def nonNegativeDeconvolution(trace, tau):
    T = len(trace)
    counts = np.zeros(T)
    counts[-1] = trace[-1]
    cutoff = math.ceil(8 * tau)
    kernel = np.exp(-np.arange(cutoff + 1)/tau)  # convolution kernel
    recent = np.full(1 + round(T / 2), np.nan).astype(int)
    recent[0] = T  # stored locations where we assigned counts
    recentIdx = 0
# the points that could potentially be assigned counts:
_delayed = np.concatenate(([0], trace[:-2]))
points = (trace[:-1] > kernel[1] * _delayed) & (trace[:-1] > 0)

# dividing these points up into runs, for speed
runStarts = np.where(points & ~(np.concatenate(([[False], points[:-1]])))[0].astype(int)
runEnds = np.where(points & ~(np.concatenate((points[1:], [False]))))[0].astype(int)
runIdx = len(runEnds) - 1

while runIdx >= 0:
    oldTop, oldBottom = 0, 0
    t = runEnds[runIdx]
    t1 = t
    accum = 0

    converged = False
    while not converged:
        if recentIdx >= 0 and recent[recentIdx] < (t+cutoff):
            t2 = recent[recentIdx] - 1
            C_max = counts[t2] / kernel[t2-t]
        else:
            t2 = min(t + cutoff, T+1) - 1
            C_max = np.inf

        b = kernel[t1-t:t2-t]
        top = np.dot(b, trace[t1:t2]) + oldTop #this is the numerator of the least squares fit for an exponential
        bottom = np.dot(b, b) + oldBottom #this is the denominator of the fit

        done = False
        while not done:
            #the error function is (data-kernel.*C)^2
            bestC = max(top/bottom, 0);  #C=top/bottom sets the derivative of the error to 0

            # does not meet nonnegative constraint. Continue to adjust previous solutions.
            if bestC > (C_max+accum):
                accum = accum + counts[t2] / kernel[t2-t]
                counts[t2] = 0
                t1 = t2
                oldTop = top
                oldBottom = bottom
                recentIdx -= 1
                done = True

            else:  # converged!
#now that we have found the MSE counts for times t<end, check if this will be swamped by the next timepoint in the run
if (t == runStarts[runIdx]) or (trace[t-1] < bestC/kernel[1]):  #%C_max won't necessarily get swamped
    if recentIdx >= 0 and t2 <= t + cutoff:
        counts[t2] = counts[t2] - (bestC - accum) * kernel[t2-t]

runStart = runStarts[runIdx]
initIdx = recentIdx + 1
recentIdx = recentIdx + 1 + t - runStart;

_skipped = 0
if recentIdx + 1 > len(recent):
    _skipped = recentIdx - (len(recent) - 1)
    recentIdx = len(recent) - 1

recent[initIdx:recentIdx + 1] = np.arange(t1, runStart + _skipped, -1)

if (runStart:runStart+t) = \[np.concatenate((trace[runStart:t], [bestC])) - np.concatenate(([0], kernel[1]*trace[runStart:t])))
done = True
converged = True
else:
    #%C_max will get swamped
    #%in this situation, we know that this point will be removed
    #%as we continue to process the run. To save time:
    t -= 1
    runEnds[runIdx] = t
    accum = accum / kernel[1]
    top = top * kernel[1] + trace[t]  # % %this is the correct adjustment to the derivative term above
    bottom = bottom * (kernel[1] ** 2) + 1  # % %this is the correct adjustment to the derivative term above

runIdx -= 1
return counts

def nndSmooth(data, hz, tau, iterFunc=None):
    tauSamples = tau * hz

    # This is the transient shape we're deconvolving against:
    # e^(x/tauSamples), for 8 times the length of tau.
    cutoff = round(8 * tauSamples)
fitted = np.exp(-np.arange(cutoff + 1) / tauSamples)

    def _singleRowNND(samples):
        result = np.copy(samples)
nanSamples = np.isnan(samples)
if np.all(nanSamples):
    pass  # No data
elif not np.any(nanSamples):
    # All samples exist, so fit in one go
    result = np.convolve(nonNegativeDeconvolution(samples, tauSamples), fitted)[:len(samples)]
else:
    # Lots of different runs of samples, fit each separately
    starts = np.where((not nanSamples) & np.isnan(np.concatenate(([1], samples[:-1]))))[0]
    ends = np.where((not nanSamples) & np.isnan(np.concatenate((samples[1:], [1]))))[0]
    for start, end in zip(starts, ends):
        tmp = np.convolve(NND(samples[start:end], tauSamples), fitted)
        result[start:end] = np.max(0, tmp[:end - start + 1])
return result

return _forEachTimeseries(data, _singleRowNND, iterFunc)

def deltaFOverF0(data, hz, t0=0.2, t1=0.75, t2=3.0, iterFunc=None):
    t0ratio = None if t0 is None else np.exp(-1 / (t0 * hz))
    tlsamples, t2samples = round(t1 * hz), round(t2 * hz)

def _singeRowDeltaFOverF(samples):
    fBar = _windowFunc(np.mean, samples, tlsamples, mid=True)
    f0 = _windowFunc(np.min, fBar, t2samples)
    result = (samples - f0) / f0
    if t0ratio is not None:
        result = _ewma(result, t0ratio)
    return result
return _forEachTimeseries(data, _singeRowDeltaFOverF, iterFunc)

def _windowFunc(f, x, window, mid=False):
    n = len(x)
    startOffset = (window - 1) // 2 if mid else window - 1
    result = np.zeros(x.shape)
    for i in range(n):
        startIdx = i - startOffset
        endIdx = startIdx + window
        startIdx, endIdx = max(0, startIdx), min(endIdx, n)
        result[i] = f(x[startIdx:endIdx])
    return result

def _ewma(x, ratio):
    result = np.zeros(x.shape)
    weightedSum, sumOfWeights = 0.0, 0.0
    for i in range(len(x)):
        weightedSum = ratio * weightedSum + x[i]
        sumOfWeights = ratio * sumOfWeights + 1.0
result[i] = weightedSum / sumOfWeights
return result

# Input is either 1d (timeseries), 2d (each row is a timeseries) or 3d (x, y, timeseries)
def _forEachTimeseries(data, func, iterFunc=None):
    if iterFunc is None:
        iterFunc = lambda x: x
    dim = len(data.shape)
    result = np.zeros(data.shape)
    if dim == 1:  # single timeseries
        result = func(data)
    elif dim == 2:  # (node, timeseries)
        for i in iterFunc(range(data.shape[0])):
            result[i] = func(data[i])
    elif dim == 3:  # (x, y, timeseries)
        for i in iterFunc(range(data.shape[0])):
            for j in iterFunc(range(data.shape[1])):
                result[i, j] = func(data[i, j])
    return result

In [28]:
def peakThreshold(trace):
    # Third Order Butterworth lowpass filter; 3hz cutoff
    # Finds the std of baseline signal to use as an amplitude threshold for peak detections
    fc = 3  # Cut-off frequency of the filter
    w = fc / (10 / 2)  # Normalize the frequency
    b, a = signal.butter(3, w, 'low', analog=True)
    z = signal.lfilter(b, a, trace, axis=0)

    # Plots to show threshold and trace with Butterworth lowpass filter
    #plt.plot(trace, linewidth=2, color='Black')
    #plt.plot(z, linewidth=2, color='orange')
    #plt.hlines(np.std(z[:]), xmin=0, xmax=z.shape[0])
    #plt.show()

    # Detect Peaks
    threshold = np.std(z[:])
    return threshold

In [73]:
def peakDetect(trace, threshold_dic, key):
    # Detect Peaks
    threshold = threshold_dic[key[:11]]

    peaks, _ = signal.find_peaks(trace, width=7, rel_height=.5, prominence=(threshold))

    width = signal.peak_widths(trace, peaks, rel_height=.5)
width10 = signal.peak_widths(trace, peaks, rel_height=.9)
return peaks, width[0], width10[0]

def signal_analysis(cell_id, gcamp, rcamp, rawG, rawR, thresholdG, thresholdR):
    print(cell[:cell.find('_Soma')])
    str_index = int(cell_id.find('Iono_'))
    threshold_cutoff = gcamp.shape[0]
    gcamp_peaks, gcamp_widths, gcamp_widths10 = peakDetect(gcamp, thresholdG, cell_id)
    rcamp_peaks, rcamp_widths, rcamp_widths10 = peakDetect(rcamp, thresholdR, cell_id)

    drug_app = np.nan
    iono_min = [np.nan, np.nan]
    iono_max = [np.nan, np.nan]
    iono_diff = [np.nan, np.nan]

    # Update: Now uses value of drug app from folder name
    # Should return -1 is no match is found for the key string 'Iono_'
    if cell_id.find('Iono_') is not -1:
        str_index = int(cell_id.find('Iono_'))
        drug_app = (int(cell_id[(str_index+5):(str_index+8)])*HZ)
        cutoffG = np.array(np.where(gcamp_peaks >= drug_app)[0])
        if cutoffG.size !=0:
            cutoffG = np.min(cutoffG)

        if cutoffG != 0:

            if cutoffG.size !=0:
                cutoffG = np.min(cutoffG)

            cutoffR = np.array(np.where(rcamp_peaks >= drug_app))
            if cutoffR.size !=0:
                cutoffR = np.min(cutoffR)

            gcamp_peaks = gcamp_peaks[:cutoffG]
            gcamp_widths = gcamp_widths[:cutoffG]
            gcamp_widths10 = gcamp_widths10[:cutoffG]

            rcamp_peaks = rcamp_peaks[:cutoffR]
            rcamp_widths = rcamp_widths[:cutoffR]
            rcamp_widths10 = rcamp_widths10[:cutoffR]

        if cell_id.find('_S') is not -1:
            if drug_app is not np.nan:
                iono_min[0] = np.min(rawG[drug_app-200:drug_app+1000]>0)
                iono_min[1] = np.min(rawR[drug_app-200:drug_app+1000]>0)

                iono_max[0] = np.max(rawG[drug_app-200:drug_app+1000])
                iono_max[1] = np.max(rawR[drug_app-200:drug_app+1000])
\[
\text{iono\_diff[0]} = \text{iono\_max[0]} - \text{iono\_min[0]} \\
\text{iono\_diff[1]} = \text{iono\_max[1]} - \text{iono\_min[1]} \\
\]

# Match Peaks and puttin them in a list of tuples (g, r)

gcamp\_matched = []
shared\_rcamp = []
shared\_gcamp = []

for g in gcamp\_peaks:
    for r in rcamp\_peaks:
        a = math.isclose(g, r, abs\_tol=15)
        if a == True:
            gcamp\_matched.append((g, r))
            shared\_rcamp.append(r)
            shared\_gcamp.append(g)

rcamp\_only = list(set(rcamp\_peaks) - set(shared\_rcamp))
rcamp\_only.sort

gcamp\_only = list(set(gcamp\_peaks) - set(shared\_gcamp))
gcamp\_only.sort

if len(gcamp\_peaks) == 0:
    g\_percent\_shared = np.nan
else:
    g\_percent\_shared = (len(gcamp\_matched) / len(gcamp\_peaks))

if len(rcamp\_peaks) == 0:
    r\_percent\_shared = np.nan
else:
    r\_percent\_shared = (len(gcamp\_matched) / len(rcamp\_peaks))

if len(gcamp\_matched) == 0:
    g\_percent\_shared = np.nan
    g\_percent\_shared = np.nan

# Integrate the area under each Traces
int\_rcamp = integrate.cumtrapz(rcamp)
int\_gcamp = integrate.cumtrapz(gcamp)

# General Stats for the cell

cell\_stats = {
    'Cell ID': cell[:,cell.find('\_Soma')],
    'GCaMP Peaks': len(gcamp\_peaks),
    'RCaMP Peaks': len(rcamp\_peaks),
    'Shared Peaks': len(gcamp\_matched),
    'GCaMP Percent Shared': g\_percent\_shared,
    'RCaMP Percent Shared': r\_percent\_shared,
    'Experiment Length (s)': gcamp\_shape[0]/HZ,
    'Drug Application': drug\_app/HZ,
    'Iono GCaMP Max': iono\_max[0],
    'Iono GCaMP Dif': iono\_diff[0],
    'Iono RCaMP Max': iono\_max[1],
    'Iono RCaMP Dif': iono\_diff[1],
    'RCaMP Total Area': int\_rcamp[-1],
}

'GCaMP Total Area': int_gcamp[-1]

cell_stats = pd.DataFrame(data=cell_stats, index=[0])

# Peak Data for Shared Peaks
shared_peak_data = pd.DataFrame()
for event in gcamp_matched:
    gindex = np.where(gcamp_peaks == event[0])[0]
    rindex = np.where(rcamp_peaks == event[1])[0]

    # Integrate Under the Curve for Area
    # Note: Area from start to peak
    g_event_start = int(event[0] - gcamp_widths[gindex])
    if g_event_start < 0:
        g_event_start = 0
    r_event_start = int(event[0] - rcamp_widths[rindex])
    if r_event_start < 0:
        r_event_start = 0

g_area = integrate.cumtrapz(gcamp[g_event_start:event[0]])
if len(g_area) is not 0:
    g_area = g_area[-1]
r_area = integrate.cumtrapz(rcamp[r_event_start:event[1]])
if len(r_area) is not 0:
    r_area = r_area[-1]

peak_stats = {
    'Cell ID': cell[:,cell.find('_Soma')],
    'Soma Loc': event[0],
    'Soma Start': event[0] - gcamp_widths[gindex][0]*HZ,
    'Soma Width': gcamp_widths[gindex][0],
    'Soma Prominence': gcamp[event[0]],
    'Soma Area': g_area,
    'Dendrites Loc': event[1],
    'Dendrites Start': event[1] - rcamp_widths[rindex][0]*HZ,
    'Dendrites Width': rcamp_widths[rindex][0],
    'Dendrites Prominence': rcamp[event[1]],
    'Dendrites Area': r_area,
    'Prominence Ratio (S/D)': (gcamp[event[1]] / rcamp[event[0]]),
    'Peak Time Diff (S-D)': ((event[0] - event[1]) * 100),
    'Start Difference (S-D)': ((event[0] - gcamp_widths[gindex]) - (event[1] - rcamp_widths[rindex]))[0] * (1/HZ),
    'Soma Start 10% Max': event[0] - gcamp_widths10[gindex][0]*HZ,
}
'Dendrites Start 10% Max': event[1] - rcamp_widths10[rindex][0] * HZ,
'Start Difference (S-D) 10% Max':
((event[0] - gcamp_widths10[gindex]) - (event[1] - rcamp_widths10[rindex]))[0] * (1/HZ)

shared_peak_data = shared_peak_data.append(peak_stats, ignore_index=True)

# Adding RCaMP peaks to the shared datatable
for event in rcamp_only:
    rindex = np.where(rcamp_peaks == event)[0]

    # Integrate Under the Curve for Area
    # Note: Area from start to peak
    r_event_start = int(event - rcamp_widths[rindex])
    if r_event_start < 0:
        r_event_start = 0

    r_area = integrate.cumtrapz(rcamp[r_event_start:event])
    if len(r_area) is not 0:
        r_area = r_area[-1]

    peak_stats = {
        'Cell ID': cell[:cell.find('_Soma')],
        'Soma Loc': np.nan,
        'Soma Start': np.nan,
        'Soma Width': np.nan,
        'Soma Prominence': np.nan,
        'Soma Area': np.nan,
        'Dendrite Loc': event,
        'Dendrite Start': event - rcamp_widths[rindex][0] * HZ,
        'Dendrite Width': rcamp_widths[rindex][0],
        'Dendrite Prominence': rcamp[event],
        'RCaMP Area': r_area,
        'Prominence Ratio (S/D)': 0,
        'Peak Time Diff (S-D)': np.nan,
        'Start Difference (S-D)': np.nan
    }

    shared_peak_data = shared_peak_data.append(peak_stats, ignore_index=True)

# Peak Data for exclusive GCaMP Peaks
gcamp_peak_data = pd.DataFrame()
for event in gcamp_only:
    gindex = np.where(gcamp_peaks == event)[0]

    # Integrate Under the Curve for Area
# Note: Area from start to peak

g_event_start = int(event-gcamp_widths[gindex])
if g_event_start < 0:
g_event_start = 0

if g_event_start < 0:
g_event_start = 0

g_area = integrate.cumtrapz(gcamp[g_event_start:event])
if len(g_area) is not 0:
g_area = g_area[-1]

peak_stats = {
    'Cell ID': cell[:,:,cell.find('_Soma')],
    'Soma Loc': event,
    'Soma Start': event-gcamp_widths[gindex][0]*HZ,
    'Soma Width': gcamp_widths[gindex][0],
    'Soma Prominence': gcamp[event],
    'Soma Area': g_area,
}

gcamp_peak_data = gcamp_peak_data.append(peak_stats,
ignore_index=True)

r_event_start = int(event-rcamp_widths[rindex])
if r_event_start < 0:
r_event_start = 0

if len(r_area) is not 0:
r_area = r_area[-1]

peak_stats = {
    'Cell ID': cell[:,:,cell.find('_Soma')],
    'RCaMP Loc': event,
    'RCaMP Start': (event-rcamp_widths[rindex][0])*HZ,
    'RCaMP Width': rcamp_widths[rindex][0],
    'RCaMP Prominence': rcamp[event],
    'RCaMP Area': r_area,
}

rcamp_peak_data = rcamp_peak_data.append(peak_stats,
ignore_index=True)

cell_stats["Prominence Ratio Mean"] =
np.nanmean(np.array(shared_peak_data["Promicence Ratio (S/D)"]))
cell_stats["Prominence Ratio SEM"] =
sem(np.array(shared_peak_data["Promicence Ratio (S/D)"]))
return cell_stats, shared_peak_data, gcamp_peak_data, rcamp_peak_data

def inspect_results(self):
masks = self.masks.copy()
trace_array = self.dff.copy()
channel1 = self.fl_image.copy()
channel2 = self.bf_image.copy()
closed = masks.copy()
contours = []
mask_values = np.unique(masks)
for i in range(len(mask_values)):
closed = masks.copy()
closed[closed != mask_values[i]] = 0
contours.append(measure.find_contours(closed, .4))

num_roi = np.unique(masks).shape[0]
color_step = (1 / (num_roi + 1))
step_color = color_step
color_list = []
for i in range(num_roi):
    plot_color = cc.glasbey[i]
    color_list.append((plot_color))
    step_color += color_step

def plot(Frame, ROI):
    contour = contours[ROI]

    plt.rcParams["figure.figsize"] = [16, 10]
closed = masks.copy()

    fig, ax = plt.subplots(2)
    ax[0].imshow(channel2[Frame, ::], cmap=plt.cm.gray)
    ax[0].imshow(channel1[Frame, ::], cmap='magma', alpha=.5)
    ax[0].plot(contour[0][:, 1], contour[0][:, 0], linewidth=4, color=color_list[ROI])
    ax[1].plot(trace_array[ROI, :], color=color_list[ROI])
    ax[1].axvline(x=Frame, color=color_list[ROI])
    plt.show()

def results(cell, RTrace, GTrace, Rdff, Gdff):
    plt.rcParams["figure.figsize"] = [16, 10]

    fig, ax = plt.subplots(2)
    ax[0].plot(RTrace.loc[cell][1], linewidth=1, color='Red')
    ax[1].plot(Rdff.loc[cell][1], linewidth=1, color='Red')
    ax[0].plot(GTrace.loc[cell][1], linewidth=1, color='Green')
```python
ax[1].plot(Gdff.loc[cell][1], linewidth=1, color='Green')
ax[0].set_ylabel('Raw Intensity')
ax[1].set_ylabel(' ΔF/F ')  
plt.show()

# Collects all the cells in the analysis data directory and groups
# Them by condition in two lists
path = os.fspath(DATA_DIRECTORY)
cells = sorted((os.listdir(path)))
WT_Cells = []
YAC128_Cells = []
for folder in cells:
    print(folder)
    if 'WT' in folder:
        WT_Cells.append(folder)
    if 'YAC128' in folder:
        YAC128_Cells.append(folder)

WT_Cells = np.unique(WT_Cells)
YAC128_Cells = np.unique(YAC128_Cells)

WT_Somas = []
WT_Dendrites = []

for cell in WT_Cells:
    if 'Soma'in cell:
        WT_Somas.append(cell)
    if 'Dendrites'in cell:
        WT_Dendrites.append(cell)

WT_Somas = WT_Somas[::-1]
WT_Dendrites = WT_Dendrites[::-1]

display(WT_Somas)
display(WT_Dendrites)

Cycle Through WT Cells to Extract Peak Data

WT_Soma_Threshold = {}
WT_Dendrite_Threshold = {}
WT_Stats = pd.DataFrame()
WT_Shared_Peaks = pd.DataFrame()
WT_GCaMP = pd.DataFrame()
WT_RCaMP = pd.DataFrame()
WT_GTrace = pd.DataFrame()
WT_RTrace = pd.DataFrame()
```
for cell in WT_Somas:
    dendrites = next(dend for dend in WT_Dendrites if (cell[:len(cell) - cell[:: -1].index('_')] in dend)

    rcamp = np.transpose(np.array(pd.read_csv(os.path.join(DATA_DIRECTORY, dendrites), sep=',', header=None)))
    gcamp = np.transpose(np.array(pd.read_csv(os.path.join(DATA_DIRECTORY, cell), sep=',', header=None)))
    rcamp = rcamp[0]
    gcamp = gcamp[0]

    # Calculate df/f and perform NND
    dffG = deltaFOverF0(gcamp, HZ)
    dffG = nndSmooth(dffG, HZ, tau=1)
    dffR = deltaFOverF0(rcamp, HZ)
    dffR = nndSmooth(dffR, HZ, tau=1)
    if len(cell)==20:
        WT_Soma_Threshold[cell[:11]] = peakThreshold(dffG)
        WT_Dendrite_Threshold[cell[:11]] = peakThreshold(dffR)

    plt.plot(dffG, color='green')
    plt.plot(dffR, color='red')
    plt.title(cell[:8])
    plt.show()

    WT_RDFF = WT_RDFF.append(pd.Series([cell, dffR], name=cell))
    WT_GDFF = WT_GDFF.append(pd.Series([cell, dffG], name=cell))

    cell_stats, peak_data, gcamp_peak_data, rcamp_peak_data =
    signal_analysis(cell, dffG, dffR, gcamp, rcamp, WT_Soma_Threshold, WT_Dendrite_Threshold)

    WT_Stats = WT_Stats.append(cell_stats, ignore_index=True)
    WT_Shared_Peaks = WT_Shared_Peaks.append(peak_data, ignore_index=True)
    WT_GCaMP = WT_GCaMP.append(gcamp_peak_data, ignore_index=True)
    WT_RCCaMP = WT_RCCaMP.append(rcamp_peak_data, ignore_index=True)

Save Results for WT to CSV

In []:

np.set_printoptions(threshold=8000)
WT_Stats.to_csv("WT_Stats.csv", index=False)
WT_Shared_Peaks.to_csv("WT_Shared_Peaks.csv", index=False)
WT_GCaMP.to_csv("WT_Soma.csv", index=False)
WT_RCCaMP.to_csv("WT_Dendrites.csv", index=False)
WT_RTrace.to_csv("WT_Dendrite_Trace.csv", index=False)
WT_GTrace.to_csv("WT_Soma_Trace.csv", index=False)
WT_RDFF.to_csv("WT_Dendrite.csv", index=False)
WT_GDFF.to_csv("WT_Soma.csv", index=False)

np.set_printoptions(threshold=10)

Display Results for WT

display(WT_Stats)
display(WT_Shared_Peaks)
display(WT_GCaMP)
display(WT_RCaMP)

Cycle Through YAC128 Cells to Extract Peak Data

YAC128_Somas = []
YAC128_Dendrites = []

for cell in YAC128_Cells:
    if 'Soma' in cell:
        YAC128_Somas.append(cell)
    if 'Dendrites' in cell:
        YAC128_Dendrites.append(cell)

YAC128_Somas = YAC128_Somas[:-1]
YAC128_Dendrites = YAC128_Dendrites[:-1]

for cell in YAC128_Somas:
dendrites = next(dend for dend in YAC128_Dendrites if cell[:8] in dend)
print(dendrites)

YAC128_Soma_Threshold = {}
YAC128_Dendrite_Threshold = {}

YAC128_Stats = pd.DataFrame()
YAC128_Shared_Peaks = pd.DataFrame()
YAC128_GCaMP = pd.DataFrame()
YAC128_RCaMP = pd.DataFrame()

YAC128_GTrace = pd.DataFrame()
YAC128_RTrace = pd.DataFrame()

YAC128_GDFF = pd.DataFrame()
YAC128_RDFF = pd.DataFrame()

for cell in YAC128_Somas:
```python
dendrites = next(dend for dend in YAC128_Dendrites if (cell[:len(cell) - cell[:-1].index('_')] in dend)
    rcamp = np.transpose(np.array(pd.read_csv(os.path.join(DATA_DIRECTORY, dendrites), sep=' ', header=None)))
    gcamp = np.transpose(np.array(pd.read_csv(os.path.join(DATA_DIRECTORY, cell), sep=' ', header=None)))
    rcamp = rcamp[0]
    gcamp = gcamp[0]
    YAC128_RTrace = YAC128_RTrace.append(pd.Series([cell, rcamp], name=cell))
    YAC128_GTrace = YAC128_GTrace.append(pd.Series([cell, gcamp], name=cell))

    # Calculate df/f and perform NND
    dffG = deltaFOverF0(gcamp, HZ)
    dffG = nndSmooth(dffG, HZ, tau=1)
    dffR = deltaFOverF0(rcamp, HZ)
    dffR = nndSmooth(dffR, HZ, tau=1)
    if len(cell)==24:
        YAC128_Soma_Threshold[cell[:11]] = peakThreshold(dffG)
        YAC128_Dendrite_Threshold[cell[:11]] = peakThreshold(dffR)
    YAC128_RDFF = YAC128_RDFF.append(pd.Series([cell, dffR], name=cell))
    YAC128_GDFF = YAC128_GDFF.append(pd.Series([cell, dffG], name=cell))

    cell_stats, peak_data, gcamp_peak_data, rcamp_peak_data =
    signal_analysis(cell, dffG, dffR, gcamp, rcamp, YAC128_Soma_Threshold,
    YAC128_Dendrite_Threshold)

    YAC128_Stats = YAC128_Stats.append(cell_stats, ignore_index=True)
    YAC128_Shared_Peaks = YAC128_Shared_Peaks.append(peak_data, ignore_index=True)
    YAC128_GCaMP = YAC128_GCaMP.append(gcamp_peak_data, ignore_index=True)
    YAC128_RCaMP = YAC128_RCaMP.append(rcamp_peak_data, ignore_index=True)

Save Results for YAC128 to CSV

np.set_printoptions(threshold=8000)
YAC128_Stats.to_csv("YAC128_Stats.csv", index=False)
YAC128_Shared_Peaks.to_csv("YAC128_Shared_Peaks.csv", index=False)
YAC128_GCaMP.to_csv("YAC128_GCaMP.csv", index=False)
YAC128_RCaMP.to_csv("YAC128_RCaMP.csv", index=False)
YAC128_RTrace.to_csv("YAC128_RTrace.csv", index=False)
YAC128_GTrace.to_csv("YAC128_GTrace.csv", index=False)
YAC128_RDFF.to_csv("YAC128_RDFF.csv", index=False)
YAC128_GDFF.to_csv("YAC128_GDFF.csv", index=False)
```

np.set_printoptions(threshold=10)

Display Results for YAC128

display(YAC128_Stats)
display(YAC128_Shared_Peaks)
display(YAC128_GCaMP)
display(YAC128_RCaMP)
ROI detection and analysis for cytosolic RCaMP-Nuclear GCaMP images code:

```python
import matplotlib.pyplot as plt
import os
import numpy as np
import pandas as pd
from tqdm.notebook import tqdm
from IPython.display import interact, interactive, fixed, interact_manual
import ipywidgets as widgets

# Change output figure size
# ...needs to be in its own cell for some reason...
plt.rcParams['figure.figsize'] = [20, 5]


To install pyneuortrace use this: pip install --upgrade
"git+https://github.com/padster/pyNeuroTrace#egg=pyneurotrace&subdirectory=pyneurotrace"

Global Settings

In [3]:

DATA_DIRECTORY = '/mnt/5404b8a5-71b7-4464-9a1e-b40cd26fac58/Data_Drive/Wissam/osfstorage-archive/Data (Images)/'

# Framerate of video in hz
HZ = 10

# Standard Deviation Threshold Multiplier
STD_THRESH = 5

# Power for Butterworth Lowpass filter
Power = 3

# Frequency cut-off
FC = 3

Collects all the cells in the analysis data directory and groups them by condition in two lists

In [4]:

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path = os.fspath(DATA_DIRECTORY)
cells = sorted((os.listdir(path)))
WT_Cells = []
YAC128_Cells = []
for folder in cells:
    if 'WT' in folder:
        WT_Cells.append(folder[:-2])
    if 'YAC128' in folder:
        YAC128_Cells.append(folder[:-2])
WT_Cells = np.unique(WT_Cells)
YAC128_Cells = np.unique(YAC128_Cells)

# Preform analysis
wt_results = pd.DataFrame(columns=['Name', 'Cell'])
for exp in tqdm(WT_Cells):
    wt_results = wt_results.append({'Name':exp,
                                   'Cell': cell.cell(DATA_DIRECTORY, exp, HZ, Power, FC, STD_THRESH)}, ignore_index=True)

# Preform analysis
yac128_results = pd.DataFrame(columns=['Name', 'Cell'])
for exp in tqdm(YAC128_Cells):
    yac128_results = yac128_results.append({'Name':exp,
                                              'Cell': cell.cell(DATA_DIRECTORY, exp, HZ, Power, FC, STD_THRESH)}, ignore_index=True)

WT_Stats = pd.DataFrame()
WT_Shared_Peaks = pd.DataFrame()
WT_GCaMP = pd.DataFrame()
WT_RCaMP = pd.DataFrame()
WT_GTrace = pd.DataFrame()
WT_RTrace = pd.DataFrame()

WT_GDFF = []
WT_RDFF = []

WT_GDFF_index = []
WT_RDFF_index = []

for i in range(wt_results.shape[0]):
    WT_GDFF_index.append(wt_results.iloc[i,1].cell)
    WT_RDFF_index.append(wt_results.iloc[i,1].cell)
WT_RDFF.append(pd.DataFrame(wt_results.iloc[i, 1].nndR))
WT_GDFF.append(pd.DataFrame(wt_results.iloc[i, 1].nndG))

WT_Stats = WT_Stats.append(wt_results.iloc[i, 1].cell_stats, ignore_index=True)
WT_Shared_Peaks =
WT_Shared_Peaks.append(wt_results.iloc[i, 1].shared_peak_data, ignore_index=True)
WT_GCaMP = WT_GCaMP.append(wt_results.iloc[i, 1].gcamp_peak_data, ignore_index=True)
WT_RCaMP = WT_RCaMP.append(wt_results.iloc[i, 1].rcamp_peak_data, ignore_index=True)

WT_GDFF = pd.concat(WT_GDFF, axis=1)
WT_GDFF = WT_GDFF.transpose()
WT_GDFF.insert(loc=0, column='Cell', value=WT_GDFF_index)
WT_RDFF = pd.concat(WT_RDFF, axis=1)
WT_RDFF = WT_RDFF.transpose()
WT_RDFF.insert(loc=0, column='Cell', value=WT_RDFF_index)

Save Results for WT to CSV

In [8]:
np.set_printoptions(threshold=8000)
WT_Stats.to_csv("WT_Stats.csv", index=False)
WT_Shared_Peaks.to_csv("WT_Shared_Peaks.csv", index=False)
WT_GCaMP.to_csv("WT_GCaMP.csv", index=False)
WT_RCaMP.to_csv("WT_RCaMP.csv", index=False)

WT_RTrace.to_csv("WT_R_Trace.csv", index=False)
WT_GTrace.to_csv("WT_G_Trace.csv", index=False)
WT_RDFF.to_csv("WT_R_DFF.csv", index=False)
WT_GDFF.to_csv("WT_G_DFF.csv", index=False)

np.set_printoptions(threshold=10)

Display Results for WT

In [9]:
display(WT_Stats)
display(WT_Shared_Peaks)
display(WT_GCaMP)
display(WT_RCaMP)

Inspect WT Results

In [10]:
def f(Experiment):
    data = wt_results.index[wt_results['Name'] == Experiment][0]
    wt_results['Cell'][data].inspect_peaks()
interact(f, Experiment=wt_results['Name'])

def f(Experiment):
    data = wt_results.index[wt_results['Name']==Experiment][0]
    wt_results['Cell'][data].inspect_results()
    interact(f, Experiment=wt_results['Name'])

Cycle Through YAC128 Cells to Extract Peak Data

YAC128_Stats = pd.DataFrame()
YAC128_Shared_Peaks = pd.DataFrame()
YAC128_GCaMP = pd.DataFrame()
YAC128_RCaMP = pd.DataFrame()

YAC128_GTrace = pd.DataFrame()
YAC128_RTrace = pd.DataFrame()

YAC128_GDFF = []
YAC128_RDFF = []

YAC128_GDFF_index = []
YAC128_RDFF_index = []

for i in range(yac128_results.shape[0]):
    YAC128_GDFF_index.append(yac128_results.iloc[i,1].cell)
    YAC128_RDFF_index.append(yac128_results.iloc[i,1].cell)

    YAC128_RDFF.append(pd.DataFrame(yac128_results.iloc[i,1].nndR))
    YAC128_GDFF.append(pd.DataFrame(yac128_results.iloc[i,1].nndG))

    YAC128_RTrace =
    YAC128_GTrace.append(pd.Series([yac128_results.iloc[i,1].cell,
                                     yac128_results.iloc[i,1].nndR], name=yac128_results.iloc[i,1].cell))

    YAC128_GTrace =
    YAC128_GTrace.append(pd.Series([yac128_results.iloc[i,1].cell,
                                     yac128_results.iloc[i,1].nndG], name=yac128_results.iloc[i,1].cell))

    YAC128_Stats = YAC128_Stats.append(yac128_results.iloc[i,1].cell_stats,
                                        ignore_index=True)
    YAC128_Shared_Peaks =
    YAC128_Shared_Peaks.append(yac128_results.iloc[i,1].shared_peak_data,
                                ignore_index=True)
    YAC128_GCaMP =
    YAC128_GCaMP.append(yac128_results.iloc[i,1].gcamp_peak_data,
                        ignore_index=True)
    YAC128_RCaMP =
    YAC128_RCaMP.append(yac128_results.iloc[i,1].rcamp_peak_data,
                        ignore_index=True)
YAC128_GDFF = pd.concat(YAC128_GDFF, axis=1)
YAC128_GDFF = YAC128_GDFF.transpose()
YAC128_GDFF.insert(loc=0, column='Cell', value=YAC128_GDFF_index)

YAC128_RDFF = pd.concat(YAC128_RDFF, axis=1)
YAC128_RDFF = YAC128_RDFF.transpose()
YAC128_RDFF.insert(loc=0, column='Cell', value=YAC128_RDFF_index)

Save Results for YAC128 to CSV

In [13]:

np.set_printoptions(threshold=8000)
YAC128_Stats.to_csv("YAC128_Stats.csv", index=False)
YAC128_Shared_Peaks.to_csv("YAC128_Shared_Peaks.csv", index=False)
YAC128_GCaMP.to_csv("YAC128_GCaMP.csv", index=False)
YAC128_RCaMP.to_csv("YAC128_RCaMP.csv", index=False)
YAC128_RTrace.to_csv("YAC128_R_Trace.csv", index=False)
YAC128_GTrace.to_csv("YAC128_G_Trace.csv", index=False)
YAC128_RDFF.to_csv("YAC128_R_DFF.csv", index=False)
YAC128_GDFF.to_csv("YAC128_G_DFF.csv", index=False)

np.set_printoptions(threshold=10)

Display Results for YAC128

In [14]:

display(YAC128_Stats)
display(YAC128_Shared_Peaks)
display(YAC128_GCaMP)
display(YAC128_RCaMP)

Inspect YAC128 Results

In [15]:

def f(Experiment):
    data= yac128_results.index[yac128_results['Name'] == Experiment][0]
yac128_results['Cell'][data].inspect_peaks()
interact(f, Experiment=yac128_results['Name'])

In [16]:

def f(Experiment):
    data= yac128_results.index[yac128_results['Name'] == Experiment][0]
yac128_results['Cell'][data].inspect_results()
interact(f, Experiment=yac128_results['Name'])