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Characterization of Excitatory Ion channels in Parkinsonian Model Sensory Neurons

Master's Thesis (30 ECTS)

Curriculum Bioengineering

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Characterization of Excitatory Ion Channels in Parkinsonian Model Sensory Neurons.

Abstract

Parkinson's disease (PD) is a neurodegenerative disease characterized by symptoms of resting tremor, rigidity, bradykinesia, and postural instability. Currently, available treatments offer control of motor symptoms but do not modify the progression of the disease. Our study focuses on the PD prodromal phase, where a variety of symptoms suggest a deficiency in sensory neuronal function. We used the immortalized sensory neuron cell line "50B11", derived from dorsal root ganglion (DRG) and a toxin, rotenone, to model parkinsonian features in sensory neurons. We determined the dose-and time-dependent effect of rotenone in 50B11 cells. Our preliminary observations suggest that overexpression of excitatory ion channels SCN 2B and HCN1, previously known to be upregulated in PD patients' skin samples have an effect on mitochondrial membrane potential in rotenone-challenged 50B11 cells. Whether excitatory ion channels SCN2B and HCN1 may contribute to sensory neuron dysfunction in PD remain to be studied.

Keywords: SCN2B, SCN5A, HCN1, Periphery, Sensory, Rotenone, 50B11, Parkinson, DRG.

CERCS: B640 Neurology, Neurophysiology

Eksitatoorsete ioonkanalite iseloomustamine parkinsonismi mudeldavates sensoorsetes neuronites.

Lühikokkuvõte

Parkinsoni tõbi on neurodegeneratiivne haigus, millele iseloomulikud sümptomid on jäsemete treemor, lihasrigiidsus, bradükineesia ehk liigutuste aeglus ja kohmakus ning raskused tasakaalu säilitamisel. Hetkel olemasolevad ravivõimalused aitavad kontrollida haiguse motoorseid sümptome, kuid ei hoia ära selle progressiooni. Oma töös keskendun Parkinsoni tõve prodoromaalsele faasile, kus mitmed sümptomid viitavad probleemidele sensoorestes neuronites. Kasutasime dorsaaljuure ganglionist pärinevat immortaliseeritud sensoorsete neuronite rakuliini 50B11 ja toksiini nimega rotenoon, et mudeldada parkonsonismile omaseid muutusi sensoorsetes neuronites. Tegime kindlaks rotenooni doosi- ja ajaspetsiifilise mõju 50B11 rakkudes. Meie esialgsed tähelepanekud viitavad, et üleekspresseerides ekistatoorseid ioonkanaleid SCN2B and HCN1, mis on varasemalt leitud ülesreguleerituna Parkinsoni haigusega patsientide nakakoest, võivad omada mõju mitokondriaalse membraani potentsiaalile rotenooniga mõjutatud 50B11 rakkudes. Kas eksitatoorsed ioonkanalid SCN2B ja HCN1 osalevad Parkinsoni haiguse korral sensoorsete neuronite düsfunktsiooni kujunemisel vajab edasist uurimist.

Võtmesõnad: SCN2B, SCN5A, HCN1, perifeerne, sensoorne, rotenoon, 50B11, Parkinson, DRG.

CERCS: B640, neuroloogia, neurofüsioloogia

TABLE OF CONTENTS

Contents

LIST OF ABBREVIATIONS

GBA-	Glucocerebrosidase
PD-	Parkinson's Disease
CNS-	Central Nervous System
PN-	Peripheral Neuropathy
RT-QuIC-	Cerebrospinal fluid real-time quaking-induced conversion
LB-	Lewy Bodies
DNA-	Deoxyribonucleic acid
ATP-	Adenosine triphosphate
mtDNA-	Mitochondrial DNA
OXPHOS-	Oxidative phosphorylation
DJI-	Protein deglycase DJ-1, Parkinson's disease protein 7
CAMp-	Cyclic Adenosine monophosphate
HCN1-	Hyperpolarization activated cyclic nucleotide-gated cation channels.
MPP+ -	1-methyl-4-phenylpyridinium
EPA -	Environmental Protection Act

DMSO-	Dimethyl sulfoxide
DRG	Dorsal Root Ganglia
TG-	Trigeminal ganglia
NGF-	Nerve growth factor
GDNF-	Glial-cell derived neurotrophic factor
E14.5	embryonic day 14.5
LTX-	Lipofectamine Reagent X
PFA-	Paraformaldehyde
PBS-	Phosphate buffered saline
DMEM-	Dulbecco's Modified Eagle Medium
TMRM-	Tetramethyl rhodamine methyl ester

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative movement disorders. In Europe, prevalence and incidence rates for PD are estimated at approximately 108–257/100 000 and 11–19/100 000 per year, respectively (Balestrino & Schapira, 2020)

PD is diagnosed clinically with a set of motor symptoms, such as tremor, rigidity, bradykinesia/akinesia, and postural instability. It has been established that many non-motor symptoms, including anxiety and depression, apathy, cognitive deficits, pain, visual and olfactory disturbances, REM-sleep disorders, weight loss, and digestive disorders can be present before the onset of motor symptoms. Currently available treatments offer good control of motor symptoms but do not modify the progression of the disease. It is suggested that a thorough characterization of the prodromal phase of PD may provide valuable knowledge for the diagnosis and intervention of PD.

Pathologically, PD is characterized by the loss of dopaminergic neurons in the pars compacta of the substantia nigra and by the accumulation of misfolded α-synuclein, which is found in intra-cytoplasmic inclusions called Lewy bodies (Poewe et al., 2017). This thesis project is part of the Dr. Pille Taba (Estonian Research Council “Team grant" project PRG957), Institute of Biomedicine and Translational Medicine in a joint project with the Tartu University Hospital to further investigate the peripheral nervous system involvement in PD. We set to find out whether peripheral neurons are more vulnerable to overexpression of excitatory ion channel subunits in PD model sensory neurons and whether their overexpression leads to mitochondrial dysfunction.

Previous work in the Tartu University Hospital had shown upregulation of some excitatory ion channels in the skin of Parkinson’s disease patients. (Planken et al., 2017a). In this thesis, I investigated the time and dose effect of rotenone on 50B11 cells and made initial observations on overexpressed excitatory ion channels in parkinsonian model sensory neurons. This knowledge may pave a way for the better treatment and diagnosis of Parkinson's disease at the earliest stages.

1. LITERATURE REVIEW

1.1 Background.

This year, 2022, marked the 205th anniversary of the publication of James Parkinson's article - An essay on the shaking palsy in 1817, where he described for the first time, in a clear and detailed manner, the state of six patients presenting the symptoms of the disease (McDonald et al., 2018). James Parkinson hoped that his description would inspire others to understand the pathology underlying the disorders and thus find an appropriate therapy.

Parkinson's disease is a neurodegenerative condition, typically characterized by symptoms of resting tremor, rigidity, bradykinesia, and postural instability (2021 Bloem). Years lived with disability and disability-adjusted life years due to Parkinson's disease increased between 1990 and 2010, and a progressive increase in the personal, societal, and economic burden associated with this disease is expected in the future as the world population ages (Yang et al., 2020)

Parkinson's disease is twice as common in men compared with women in most populations. It is suggested that protective effect of female sex hormones, a sex-associated genetic mechanism, or a sex-specific difference in exposure to environmental risk factors might explain this male preponderance (Figure 1) (van den Eeden, 2003). However, in few populations, including one study from Japan, little sex differences were observed (Periquet et al., 2003). The incidence seems to also vary within subgroups defined by race, ethnicity, genotype, or environment (Yang et al., 2020). For example, PD may be less common in African Americans and Asians in the United States. Still, the systematic race-specific incidence has not been investigated in other multiracial populations, and societal rather than biological causes might underlie these findings (Chillag-Talmor et al., 2011). Geography and race are often related, and it might be challenging to determine the relative contribution of each to the risk of developing Parkinson's disease.

Gene-environment interactions seem to modify the risk for sporadic Parkinson's disease. For example, the prevalence of Parkinson's disease is significantly greater in individuals exposed to certain environmental factors, such as pesticides and traumatic brain injury, and lower in smokers or caffeine users (Hu et al., 2022). The pesticides Paraquat and Rotenone which causes oxidative stress and inhibit mitochondrial complex I function, induce a loss of nigral dopaminergic neurons and behavioural changes associated with human PD (Narayan et al., 2017)

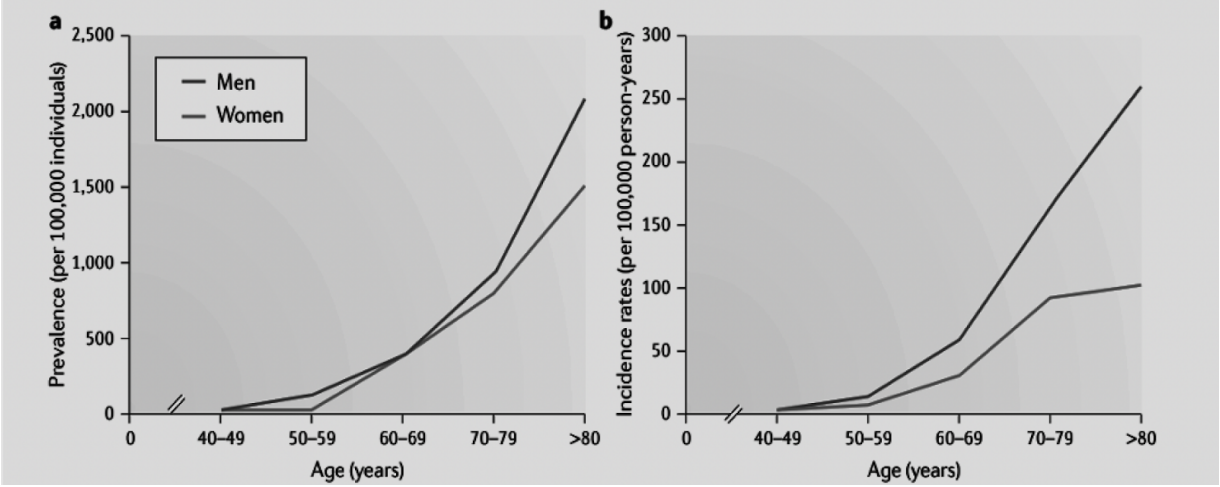

Figure 1. A. Prevalence of PD in men and women per 100,000 individuals. B. Incidence rate of Parkinson's disease per 100,000 person-years. Image adapted and modified from (Draoui et al., 2020a).

1.2 Symptomatology of Parkinson's Disease.

Parkinson's disease is diagnosed based upon clinical presentation. This stage is marked primarily by motor symptoms of akinesia, bradykinesia, and resting tremor (Duty & Jenner, 2011). Akinesia is the most important of the significant signs of PD, defined by the difficulty of initiating a movement and resulting in a decrease in the frequency of voluntary acts. This difficulty is often associated with bradykinesia, a slowing down of movements. Speech ability is also affected by the rareness of words and poor articulation and a

monotonous and low voice (Rodriguez-Oroz et al., 2009). Tremor is the most identified initial sign during the symptomatic phase. It concerns mainly the distal parts of the upper limbs when at rest and is characterized by regular oscillations of low frequency and amplitude. It also consists of adduction of the fingers and hands (Rodriguez-Oroz et al., 2009).

Non-motor symptoms of PD include anxiety and depression, apathy, cognitive deficits, visual and olfactory disturbances, REM, sleep disorders, weight loss, and digestive disorders (Draoui et al., 2020b). Non-motor symptoms of PD have remained for a long time underestimated and undervalued from a clinical perspective. Now, they are highly recognized and are of great importance because of their impact on the quality of life of PD patients.

A recent study placed the initial evolution of PD into three putative periods based on motor phenotype and severity of nigrostriatal degeneration. They were first divided into three different stages (Figure 2). Initially, there is a silent motor period when nigrostriatal loss begins, but this does not disturb basal ganglia output activity (Blesa et al., 2022) (Poewe et al., 2017). This period is followed by a prodromal motor period, where subtle focal motor manifestations are associated with asymmetric dopaminergic depletion in the caudal putamen (Berg & Postuma, 2018). Finally, the manifest period, where the disease is often diagnosed, is typically characterized by the presence of cardinal motor features in one body part (most often the upper limb) on one body side (Blesa et al., 2022). It is also recognized that some non-motor manifestations, such as hyposmia and constipation, are present in a majority of PD patients at diagnosis and are prognostic of faster disease progression (Blesa 2022).

The gut-brain axis is an important topic in Parkinson's disease research and in an attempt to decipher its role in disease pathogenesis, several animal models have been developed. Most of these models tried to reproduce Braak's hypothesis (Rietdijk et al., 2017) by showing that the pathological process could spread from the gut to the brain (bottom-up scenario). Interestingly, other groups showed that a top-down scenario could also occur,

and that 6-hydroxydopamine-induced nigrostriatal denervation was sufficient to induce significant changes in the gastrointestinal tract (Leclair-Visonneau et al., 2020).

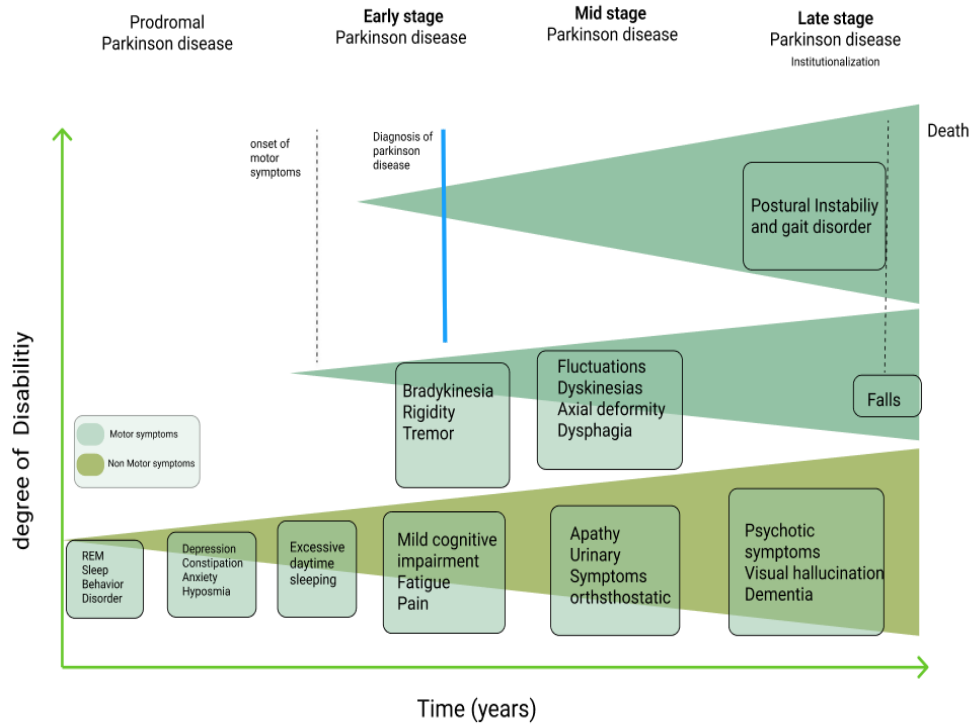

Figure 2. The symptomology of Parkinson's disease is divided into a silent non-motor period, a prodromal non-motor period before diagnosis, and a manifested period following diagnosis. Image adapted and modified from (Poewe et al., 2017).

1.3 Peripheral Nervous system and the Manifestation of Parkinson's Disease on the skin of patients.

In PD patients, characteristic inclusions, termed Lewy bodies, containing misfolded α-synuclein proteins are found in the neurons of the substantia nigra, olfactory bulb, locus coeruleus, pontine tegmentum, hypothalamus, limbic system, and amygdala nuclei. Outside the central nervous system (CNS), sympathetic and parasympathetic pre- and postganglionic nerve endings in the heart, the digestive tract, and the skin also show α-synuclein aggregates (S. Xu et al., 2006). It is still unclear whether these aggregates display similar toxicity in peripheral cells to that observed in the CNS, but multiple autonomic

dysfunctions suggest a mechanism for the widespread cell damage associated with α-synuclein aggregates (Rodríguez-Leyva et al., 2014).

Typically, neuropathies cause numbness, tingling, muscle weakness, and pain in the affected area due to damage or dysfunction of one or more nerves. Neuropathy, often called peripheral neuropathy (PN), indicates a problem within the peripheral nervous system. The peripheral nervous system is the network of nerves outside the brain and spinal cord. Neuropathy can affect one nerve (mononeuropathy) or nerve type, a combination of nerves in a limited area (multifocal neuropathy), or many peripheral nerves throughout the body (polyneuropathy). Neuropathy is very common, and the condition affects people of all ages, however, older people are at increased risk. About 8% of adults over 65 years of age report some degree of neuropathy. Other than age, in the United States, some of the more common risk factors for neuropathy include diabetes, metabolic syndrome (high blood pressure, high cholesterol, obesity, diabetes), and heavy alcohol use.

Three nerve types form the peripheral nervous system. Motor nerves innervate and control skeletal muscle activity. Autonomic nerves are responsible for body functions, some of which occur outside direct control, such as breathing, digestion, heart rate, blood pressure, sweating, bladder control, and sexual arousal. Sensory nerves carry messages from the five senses (sight, hearing, smell, taste, and touch) through the spinal cord to the brain. In Figure 3, the sensory pathway from the periphery to the central nervous system is shown.

Several studies have tried to determine if PN is more common among people with PD as opposed to people without PD. PN is a relatively common condition in the general population, which makes it difficult to ascertain whether it is even more common among people with PD. A recent study tried to clarify any evidence of peripheral nervous system involvement in idiopathic PD. They used a systematic computer-based literature search to conduct the prevalence of large fiber PN in PD and discovered that large fiber PN in PD is in most cases distal, symmetrical, axonal, and predominantly sensory. In their findings, they also saw few reports of chronic idiopathic demyelinating polyneuropathy and very occasional cases of acute neuropathies. Although nerve conduction studies have been

performed in most of their studies, they included only a limited number of nerves, mainly in the lower limbs. (Zis et al., 2017)

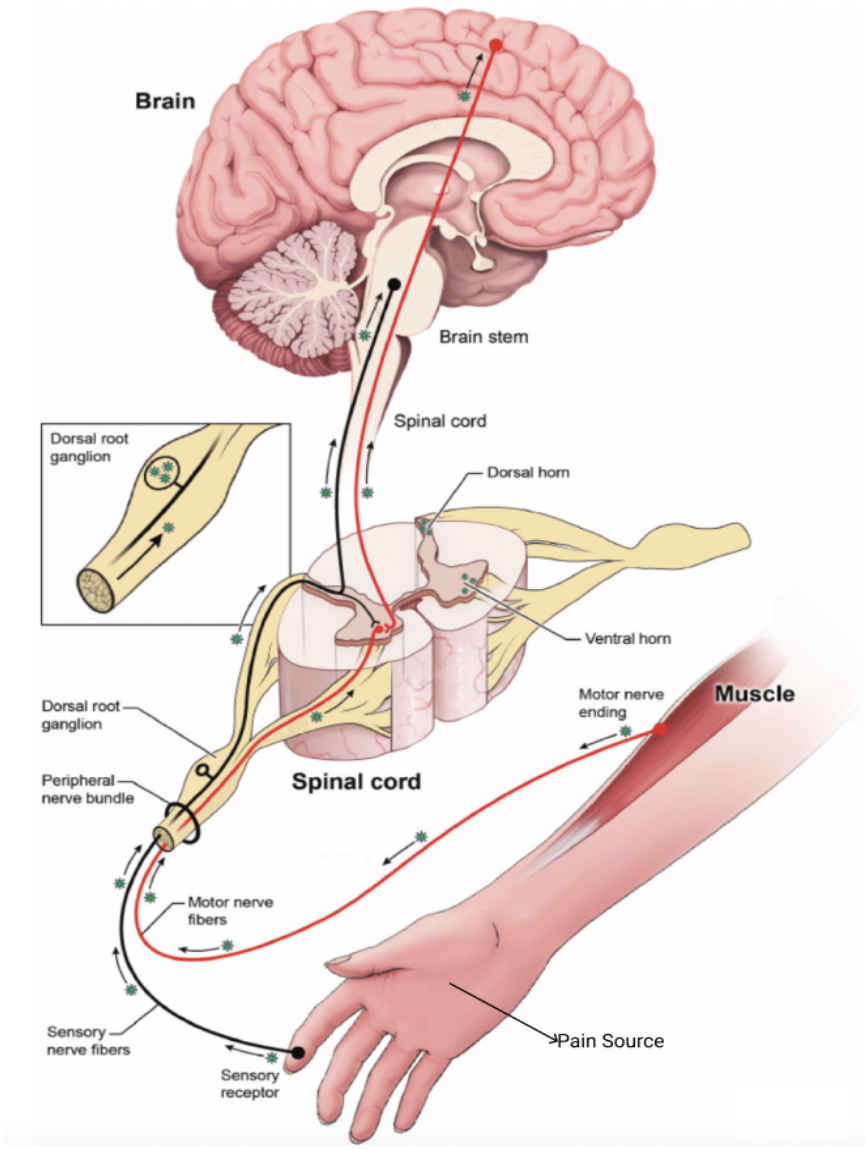

Figure 3. The cell body of each sensory neuron in the dorsal root ganglion has a long axon that extends from the dendrites located in the skin (receptors), muscles, tendons, joints, and internal organs to the cell body and another than extends from the cell to the spinal cord. These cells transduce touch (various kinds), stretch, temperature, and pain nociceptive signals. The image was taken and adapted from (Swanson & McGavern, 2015).

Animal models suggest that α-synuclein may be involved in axonal degeneration following traumatic peripheral nerve injury (Comi et al., 2014). PD patients display loss of epidermal nerve fibers and Meissner corpuscles, which correlates with sensory dysfunction (Comi et al., 2014). PD patients also show several skin symptoms including cutaneous neuropathy, seborrhea, hyperhidrosis, and impaired wound healing (Kuzkina et al., 2021). A further link to skin involvement in PD has been provided by studies demonstrating the presence of cutaneous denervation and α-synuclein deposits in dermal somatosensory and autonomic nerve fibers (Wang et al., 2013). Importantly, α-synuclein RT-QuIC assays of PD patient skin biopsies supported the clinical diagnosis of PD with a diagnostic accuracy of 88.9% and showed a high degree of inter-rater agreement between two laboratories (92.2%)(Kuzkina et al., 2021). The assay, which essentially measures the ability of alpha synuclein in skin to cause the aggregation of monomeric apha synuclein in vitro, revealed a higher seeding activity of α-synuclein in patients with longer disease duration and more advanced stages, and correlations were observed with the presence of REM sleep behaviour disorder, cognitive impairment, and constipation (Kuzkina et al., 2021).

1.4 Insights on the Role of Mitochondrial dysfunction and Oxidative stress in PD.

Mitochondrial dysfunction plays a key element in the pathogenesis of Parkinson's disease (Bose & Beal, 2016). Mitochondria are ubiquitous in eukaryotic cells. They have multiple functions and are host to numerous biochemical pathways. Two of their most important functions are related to the production of ATP by oxidative phosphorylation and the mediation of signals for apoptotic cell death. Mediation of signals and the role of mitochondria in apoptosis are particularly important in the context of PD. Mitochondria are notable for also containing their own DNA. Mitochondrial DNA (mtDNA) is a small circular double-stranded molecule with 16 493 bases long, encoding two ribosomal RNAs, 22 transfer RNAs, and 13 proteins. These 13 proteins are all part of the oxidative phosphorylation (OXPHOS) system for generating energy by aerobic metabolism. MtDNA remains dependent on the nucleus to produce proteins involved in its transcription,

translation, replication, and repair. Mutations in nuclear genes encoding proteins involved in mtDNA maintenance, e.g., mtDNA polymerase gamma, can cause a variety of human disorders including late-onset PD (Schapira, 2007).

In animal models, injection of several toxins that impair mitochondrial function replicates features of Parkinson's disease neuropathology (Bose & Beal, 2016). When mitochondrial transcription factor A, which is essential for mitochondrial DNA expression, is selectively depleted in dopaminergic neurons of MitoPark mice, mitochondria in dopaminergic neurons in the substantia nigra develop a defective electron transport chain, leading to neuronal degeneration in adulthood (Zheng et al., 2010). Moreover, rotenone, a piscicide, is a specific inhibitor of complex I of the electron transport chain and has been used widely to create animal models of PD (Innos & Hickey, 2021).

Oxidative stress, because of mitochondrial dysfunction, is increased in the brain tissue of patients with Parkinson’s disease, but it is, however, arguable if it occurs early or late during the demise of neurons (Bose & Beal, 2016). Mutations in DJ1 (also known as PARK7), encoding a putative antioxidant, which causes early-onset autosomal recessive Parkinson’s disease is associated with increased cellular oxidative stress (Jin & Youle, 2012). DJ1 deficient mice show increased protein oxidation in stressed nigral dopaminergic neurons. Nigral dopaminergic neurons have been suggested to be particularly vulnerable to metabolic and oxidative stress. It has been also proposed that low levels of α-synuclein are normally present in mitochondria and accumulation of the protein inside mitochondria leads to mitochondrial complex I deficits and oxidative stress (Pissadaki & Bolam, 2013). Notably, rotenone also induces oxidative stress through its inhibition of electron cycling via complex I (Innos & Hickey, 2021).

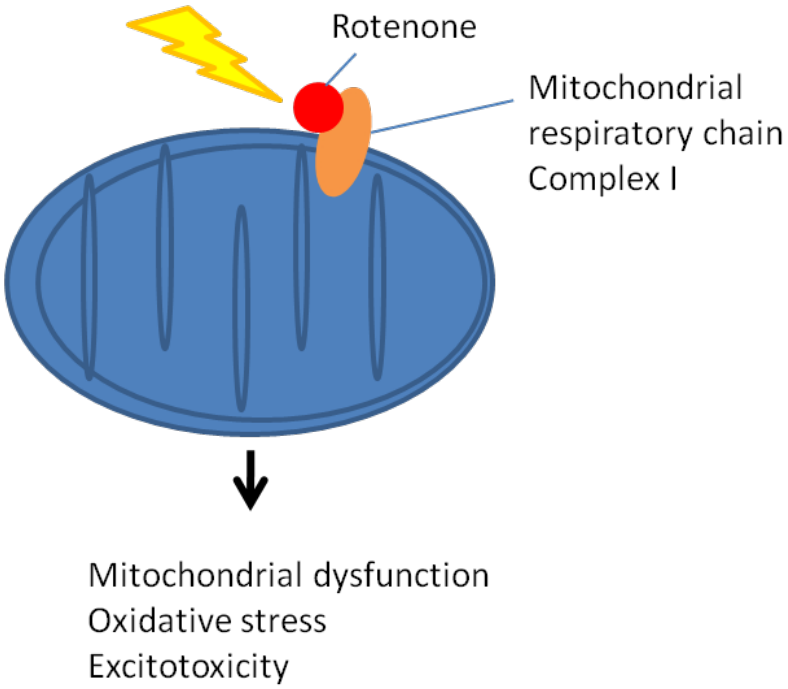

Figure 4. The mechanism of action of Rotenone. Blocks mitochondrial respiratory chain complex I thereby inducing mitochondrial dysfunction and oxidative stress. Image created by Maili Jakobson.

1.5 Ion Channels and the Expression of SCN2B, SCN5A & HCN1 In Neurodegeneration.

Our recent work highlighted several gene changes in the skin of Parkinson's patients, including several ion channels or ion channel regulatory subunits, SCN2B, SCN5A, and HCN1 (Planken et al., 2017). Structurally, HCN channels resemble K^+ channels, consisting of four domains with each domain consisting of six transmembrane segments. In each domain, the S4 segment is positively charged and represents the voltage sensor. As the name suggests, these channels are activated by membrane hyperpolarization, and once opened these channels flux K^+ and Na^+, which results in a depolarization of the membrane due to a reversal of potential of around –30 mV (João Quevedo, 2019). Their voltage sensitivity can be modulated by cyclic adenosine monophosphate (cAMP) binding to a site within the C-terminus, affecting the voltage dependence of activation. The channel was

first discovered in cardiac sinoatrial node cells where its activation drives pacemaker cells to threshold voltages and activation of Ca^{2+} channels and action potential firing. In the brain, HCN channels are expressed in dendrites, somas, and axon terminals of neurons and they are likely to generate rhythmic activity, modulating network excitability. Action potentials in nociceptive afferents are affected by the activity of hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN) family and are directly involved in neuropathic pain which is a chronic pain state caused by peripheral or central nerve injury because of acute damage or systemic diseases (Liu et al., 2017)

SCN2B with a molecular weight of 24,326 Da (Figure 5) is crucial in the assembly, expression, and functional modulation of the heterotrimeric complex of the SCN sodium channels, formed by the alpha subunits. Typically, alpha subunits are accompanied by one or two beta subunits, which served to stabilize the alpha subunits (Goldin, 2007). It is primarily expressed in the brain followed by the proximal digestive tract and muscle (https://www.proteinatlas.org/ENSG00000149575-SCN2B/tissue). SCN2b is expressed in DRGs (Lopez-Santiago et al., 2006) and reducing the protein expression of SCN2b ameliorates neuropathic pain in experimental animals (Sakai et al., 2013; Pertin 2005).

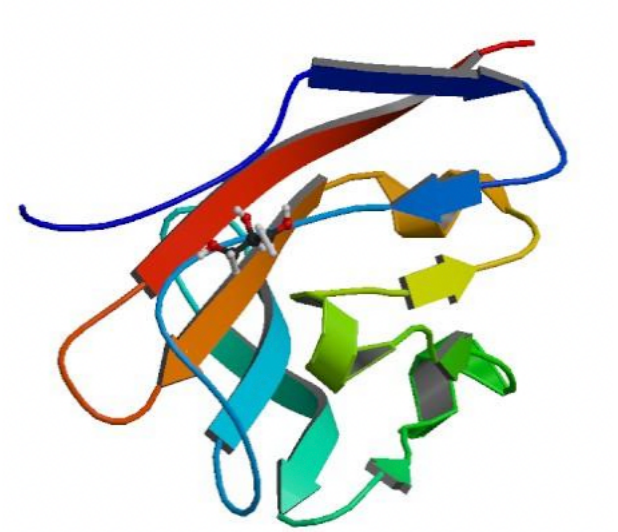

Figure 5: Representative image of SCN2B 5FEB-A+30-151 (human). Created by cell signaling technology under the creative common license and attribution- Noncommercial ShareAlike 3.0 updated license.

SCN5A is an integral membrane protein and tetrodotoxin-resistant voltage-gated sodium channel subunit (Verstraelen et al., 2015) and it is expressed in DRGs (Kerr et al., 2007). This protein is found primarily in cardiac muscle and is responsible for the initial upstroke of the action potential in an electrocardiogram. Defects in this gene are a cause of long QT syndrome type 3 (LQT3), an autosomal dominant cardiac disease. The SCN5A protein is composed of four homologous transmembrane domains (DI-DIV) with six transmembrane segments (S1-S6) in each section (Beyder & Farrugia, 2016). The four sections S1-S4 domains form the channel's voltage-sensing region, and the four S5-S6 domains with the intervening loop region form the central pore region and selective filter.

1.6 The Rotenone Model for Parkinson's Disease.

Rotenone is a chemical compound that inhibits mitochondrial respiratory chain complex I (Figure 4, 7). It induces mitochondrial dysfunction and eventually cell death in mammals

(Innos and Hickey 2021). Subacute levels of Rotenone can be used to induce PD-related molecular changes in cells. Rotenone is typically used to define the specific activity of complex I (Greenamyre et al., 2003). It is also a commonly used, naturally occurring, organic pesticide, and it is used in lakes and reservoirs to kill nuisance fish. Because it is extremely lipophilic, it crosses biological membranes easily and is independent of transporters (unlike MPP+), and it gets into the brain very rapidly (Greenamyre et al., 2003). As such, it is well-suited for inducing a systemic inhibition of complex I in experimental animals. Some in vivo rotenone models also reproduce peripheral signs of PD, such as reduced intestinal motility and peripheral α-synuclein aggregation, both of which are thought to precede classical signs of PD in humans, such as cogwheel rigidity, bradykinesia, and resting tremor (Innos & Hickey, 2021).

Rotenone is not a cause of PD, but it is rather regarded as a risk factor, suggesting that only some individuals will develop PD from continuous use and exposure. Occupational human exposure to rotenone is presumably via dermal and inhalation routes. The Environmental Protection Act shows a rate of absorption from skin of 10% per day, and for acute toxicity, this route is considered less important and less dangerous than inhalation (Dick et al., 2007). However, another important factor is the duration of exposure as in humans, several years of frequent use of pesticides increases the risk of subsequent development of PD. The EPA has assigned rotenone as category I for oral and inhalation routes (high acute toxicity). Inhalation will also result in enteral absorption, depending upon particle size (Innos & Hickey, 2021)

Rotenone is stable in solid-state and is known to degrade in water (Ling, 2003) Importantly, exposure to water, light, or excess temperatures degrades rotenone (Innos and Hickey, 2021). Animal models suggest the importance of dissolution and particle size. Animals exposed to rotenone through contact or via intranasal administration where DMSO was used as the vehicle developed parkinsonian symptoms and neuropathology compared to cases where no parkinsonism was observed when using saline with sonication as the vehicle (Innos and Hickey 2021)

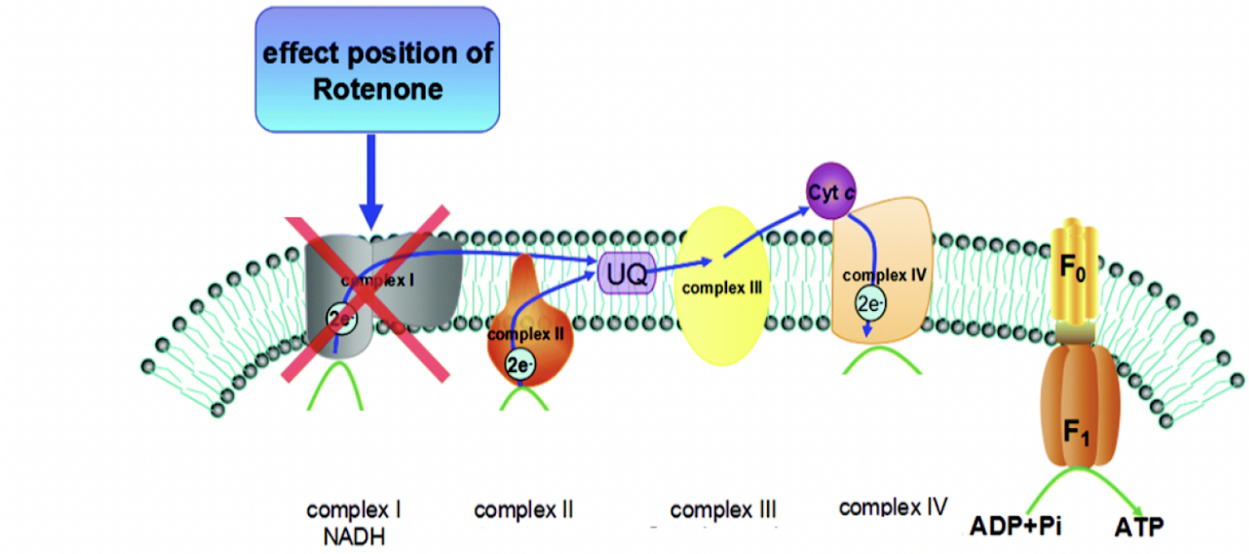

Figure 7. A schematic diagram showing the effect position of rotenone inhibiting complex I pathways, which are crucial for cell survival. Image adapted and modified from (X.-L. Xu et al., 2016).

1.7 Immortalized sensory neurons

Sensory neurons are grouped in the dorsal root ganglia (DRG) or the trigeminal ganglia (TG) and have pseudo unipolar processes that divide into two axons, one innervating targets in the periphery and the other projecting centrally in the spinal cord dorsal horn (DRG) or, in the case of the trigeminal sensory nuclear complex, to the brain stem (TG).

These sensory neurons, expressing an abundance of channels and receptors, monitor the environment and convey information including nociception, temperature, and mechanosensation to the central nervous system (CNS) (Haberberger et al., 2020a). A

substantial proportion of pain research focuses on nociceptors, the subgroup of DRGs involved in nociception and pain signaling, increasing the understanding of their basic functional properties and their critical roles in the development and maintenance of chronic pain (Rugiero & Wood, 2009), as well as identifying potential targets for therapeutic intervention. In addition to nociceptors, DRGs also contain cell bodies of non-nociceptive temperature sensing, mechanosensory, and chemoreceptive neurons, as well as multiple non-neuronal cell types such as immune cells and endothelial cells. The heterogeneous population of sensory neurons within the DRG can be subdivided into multiple subpopulations based on morphological, neurochemical, electrophysiological, and transcriptional characteristics (Rugiero & Wood, 2009).

Complementing *in vitro* models of isolated and dissociated primary DRG neurons, immortalized cell lines, despite their limitations, have enabled important advances in understanding sensory neuron function. They retain significant phenotypic similarities to primary cultures in addition to greater suitability for high throughput assays due to high consistency, reducing the number of experimental animals required as well as reducing other costs normally associated with isolation and dissociation of primary cells. In the last 3 decades, only a handful of DRG-derived immortalized sensory neuronal cell lines have been generated (Table 1). These include the hybrid cell lines F-11, ND-C, and ND7/23 (Rugiero & Wood, 2009), the mouse cell line MED17.11, the rat cell line 50B11, and human HD10.6 cells (Raymon et al., 1999b). As expected, each of the cell lines has its own unique nociceptor-like characteristics.

Immortalized sensory neuron cell lines present valuable tools for the investigation of many aspects of neurobiology, including nociception. All the cell lines created thus far share a multitude of characteristics with both embryonic and adult mammalian DRG neurons. However, as is the case with most cell lines, some major characteristic differences exist that must be carefully considered prior to their use. Recognizing the distinct functional, morphological, and neurochemical characteristics of each immortalized sensory cell line compared to native neurons is one crucial step that enables judicious interpretation of experimental results.

50B11 neurons were derived from rat dorsal root ganglia (DRG) and were developed with the purpose of testing neuroprotective strategies for peripheral neuropathies targeting nociceptive cells (Chen et al., 2007). As a result of their initial characterization and subsequent publication, 50B11 cells are comparable to a subclass of small, unmyelinated, C-fiber nociceptors that contribute to pain signaling via the transduction of high threshold signals from the periphery (Marmigère & Ernfors, 2007). In addition to the F-11 and ND7/2 cells derived from DRGs/neuroblastomas, cells from these lines also expressed markers for unmyelinated and myelinated neurons. In 50B11 cells, a replication rate of about 36 h is obtained on uncoated plastic dishes, and they have been examined to investigate cell toxicity (Chen et al., 2007).

Classification of maintenance of Dorsal Root Ganglion cell lines

	F-11	ND7/23	ND-C	50B11	MED 17.11	HD10.6
Species of origin	Rat DRG E13-14 x mouse neuroblastoma cell line N18TG2	Rat DRG E13-14 x mouse neuroblastoma cell line N18TG2	Rat DRG E13-14 x mouse neuroblastoma cell line N18TG2	Rat DRG E14.5	rat DRG E14.5	Human DRG embryonic
Culture surface and matrix composition	plastic Poly-D-lysine.	Glass/plastic. Poly-D-lysine.	Glass/plastic, collagen type	Glass/plastic No matrix	Poly-L-ornithine.	Glass - Matrigel-coated. poly-D-lysine.
Differentiation agents used	CAMP, retinoic acid, and NGF. BDNF. NT	db-cAMP retinoic acid NGF	db-cAMP	Forskolin, NGF. GDNF. estrogen, angiotensin II	Forskolin. NGF. GDNF. bFGF. Y-27623	Forskolin. NGE GDNE, CNTF, heregulin
Noinceptor-related molecules and responses	Present. Predominantly in differentiated cells	Some molecules and channels present but no Functional TrpV1, or TrpA1 channels.	Present. Predominant in differentiated cells.	Present, predominantly in differentiated cells	Present, Predominantly in differentiated cells.	Present, predominantly in differentiated cells.
Endogenous on Channel expression	Voltage-gated Ca?+ and Na channels present. Differentially expressed In undifferentiated and differentiated cells	Voltage-gated Ca+ and Na channels present different y expressed in Differentiated and undifferentiated cells	Voltage-gated Ca+ and Na channels present different y expressed in Differentiated and undifferentiated cells	Indirect evidence for voltage-gated Ca2+ Channels, no Electrophysiological studies conducted	direct evidence for voltage-gated Ca2+ channels. no Electrophysiological studies conducted	voltage-gated Ca?+ and Na+ channels present in Differentiated cells
Primary reported use	Investigation or differentiation and neurite outgrowth	Voltage-gated Ca + and Na channels present differently expressed in differentiated cells	Investigation mechanosensitivity.	Investigations of toxicity	Only one descriptive publication	Investigation of virus transfection
experimental consideration	After 5 passages no response to gabapentin Loss of opioid receptors	Mouse and rat mRNAs and has on some proteins present and expressed	Has only some characteristics of nociceptors.	Electrophysiological characteristics unknown	Not available	Limited description due to the low number of publications

Table 1: Immortalized dorsal root ganglion-derived cell lines including their origin, neurochemical characteristics including sensory and nociceptor-related molecules, and endogenous ion channels, presented in a table that summarizes different methodologies in relation to culturing different cell types. 50B11 is highlighted in green. Table adapted from (Haberberger et al., 2020c).

2. EXPERIMENTAL WORK

2.1. Aims of the Thesis

The aim of this thesis is to investigate whether peripheral neurons are more vulnerable to overexpression of excitatory ion channel subunits in PD model sensory neurons and whether their overexpression leads to mitochondrial dysfunction.

The Objectives of this thesis are as follows:

1. To determine the optimal rotenone concentration for use in 50B11 cells.

2. To characterize the effects of overexpressing excitatory ion channels on mitochondrial membrane potential.

2.2. Material and Methods

2.1. Cell lines

We used the 50B11 cell line, which is an E14.5 rat dorsal root ganglia (DRG)-derived immortalized sensory neuron cell line. The 50B11 cell line was acquired as a kind gift from Dr. Ahmet Höke's laboratory at Johns Hopkins University. 50B11 cells were maintained in a growth medium consisting of Neurobasal medium (Life Technologies, Gibco) supplemented with 10% fetal bovine serum, FBS (Life Technologies, Gibco), 1% B27 serum supplement (Life Technologies, Gibco), 20 mM D-glucose (Life Technologies, Gibco), and 1% glutaMAX™ media supplement (Life Technologies, Gibco).

2.2 Transfection

50B11 cells were transfected with Lipofectamine LTX transfection reagent according to the manufacturer´s instructions (Invitrogen). Briefly, one day before transfection, 50B11 cells were seeded in four-quadrant glass-bottomed cell culture dishes (CellVis, Mountain View, CA). After 24h, the culture medium was replaced with the Optimem medium, and

cells were transfected with up to 500ng of plasmid DNA, mixed with Lipofectamine reagents in Optimem medium for 4 hours. After that, the cell culture medium was replaced with a normal growth medium. Plasmids were a kind gift from Prof. Noriyuki Nakashima (HCN1) of Kurume University (Japan), or from the DNASU Plasmid Repository (HsCD00860587 (SCN5a); HsCD00512658 (SCN2b).

2.3 Fixation

50B11 cells were fixed with fresh 4% PFA in PBS + 250uM sucrose. Briefly, cells were pre-fixed by adding 4% PFA in PBS + 250uM sucrose solution into the cell culture medium, 2:5 ratio of fixative to medium for 2 minutes at room temperature on a horizontally rotating shaker at a rotating speed of 75 rpm. Media was then removed and cells were fixed in 4% PFA in PBS + 250uM sucrose solution, 500uL per quadrant, for 10 minutes at room temperature on the shaker. Cells were washed three times with 500uL/quadrant 1xPBS at room temperature on the shaker for 5 minutes and stored at +4 degrees in 1xPBS for processing.

2.4 Hoechst staining

Fixed cells were washed three times with PBS for 5 minutes each and then nuclei were stained using Hoechst (1ug/ml) in PBS for 5 mins. Following a final wash, a drop of fluorescence mounting medium was added and cells were stored at +4C until imaging.

2.5.Treatment with Rotenone

A stock solution of 200 μM rotenone was prepared in dimethyl sulphoxide (DMSO) and then diluted in DMEM to final concentrations. The DMSO concentration in the culture medium did not exceed 0.01%. For each treatment, fresh rotenone solution stock was used to avoid its deterioration. Cultures were treated with 1nM, 10nM, 100nM, and 500 nM rotenone for 24h, 48h, and 72h respectively to investigate the dose- and time-dependent toxicity.

2.6 Confocal microscopy imaging

Live cell imaging was performed using in the 4-chamber dishes, using Immersol 518F immersion oil for fluorescence microscopy. Live cell imaging was performed at 37°C. Stained cell culture dishes were imaged at room temperature. Images were taken using Carl

Zeiss Laser Scanning Microscope, LSM 780 using 10x or 40x objectives. Numerical Aperture: 0.25, Working Distance: 7.45 mm (Excitation wavelengths: 488 nm, emission signal at 515 nm), and images were processed using Zen software (2010) and ImageJ.

2.7. Mitochondrial membrane potential analysis in live cells

Imaging of mitochondrial membrane potential was performed in live GFP- and channel-transfected 50B11 cells. Mitochondrial membrane potential was visualized using MitoProbe TMRM Assay Kit Invitrogen from Thermo Fisher (LOT 2326036, ref 134361). The kit contains tetramethylrhodamine methyl ester (TMRM) for the detection of mitochondrial membrane potential state. Briefly, cells were loaded with 10nM TMRM reagent in a regular culture medium at 20 minutes in the incubator and imaged at 37°C in the culture medium. Up to 10 images per quadrant were imaged.

Images showing the nucleus and mitochondria was identified (Figure 13). GFP-transfected cells were outlined using the freehand tool in Fiji image analysis software and collected within the ROI (region-of-interest) manager of Fiji. These ROIs were then used to identify associated mitochondria within the same cell for estimation of mitochondrial membrane potential in cells that expressed channels of interest. This work is ongoing.

2.8. Data Processing and Statistical Analysis

Images were converted to 8-bit TIFF format, thresholded, and the mean and percentage areas of the nuclei were calculated. Transfected cells with matching mitochondria had their surface area determined, and untransfected cells were also measured and used as controls on Fiji software (Image J, NIH). Statistical analyses was performed using GraphPad Prism 9.3.1 software. Differences were considered significant at $P \leq 0.05$.

3. RESULT AND DISCUSSIONS.

3.1. Effects of rotenone on the survival of immortalized 50B11 cells.

Cells were treated with increasing concentrations of rotenone and then fixed at 24hrs, 48hrs, or 72hrs, for Hoechst staining and confocal microscopic imaging.
Our results indicated that rotenone was toxic to 50B11 cells in a dose- and time-dependent manner (Figure 8).

At 1nM and 10nM respectively, cells were minimally affected. Indeed, the mean percentage area occupied by nuclei increased between 24hrs and 48hrs in cells treated with 10nM rotenone ($p<0.05$).

The reduction in nuclei caused by 100nM rotenone at 24hrs amounted only to 16% compared with control, proving that the concentration was not sufficient to induce cell death. By 48hrs, cells were reduced by an additional 10% and cells treated with this concentration (100nM) did not show an increase percentage area occupied by nuclei between 24hrs and 48hrs.

The addition of 500 nM rotenone to cultures for 24 h decreased the survival of neurons by 26% (Figure 9, $p<0.05$) while a longer treatment for 72h progressively decreased the number of cells to 50% of control-treated cells ($p<0.05$).

As shown in Figures 10 and 11, cells showed resistance to rotenone treatment in lower concentrations (1-10nM) and a gradual compromise of cells at higher concentrations was observed (effect of treatment $F (4, 34) = 14,20<$, $p<0.0001$). Moreover, there was a clear effect of time ($F (1,596, 47,87) = 12,87$, $p<0.0001$), with longer times revealing more toxicity.
In order to model PD, which is a chronic disease, and not an acute disorder, we chose a concentration of 100nM, at a timepoint of 48 hrs (Figures 10 and 11) for future experiments. At this timepoint and concentration, frank cell death was minimal.

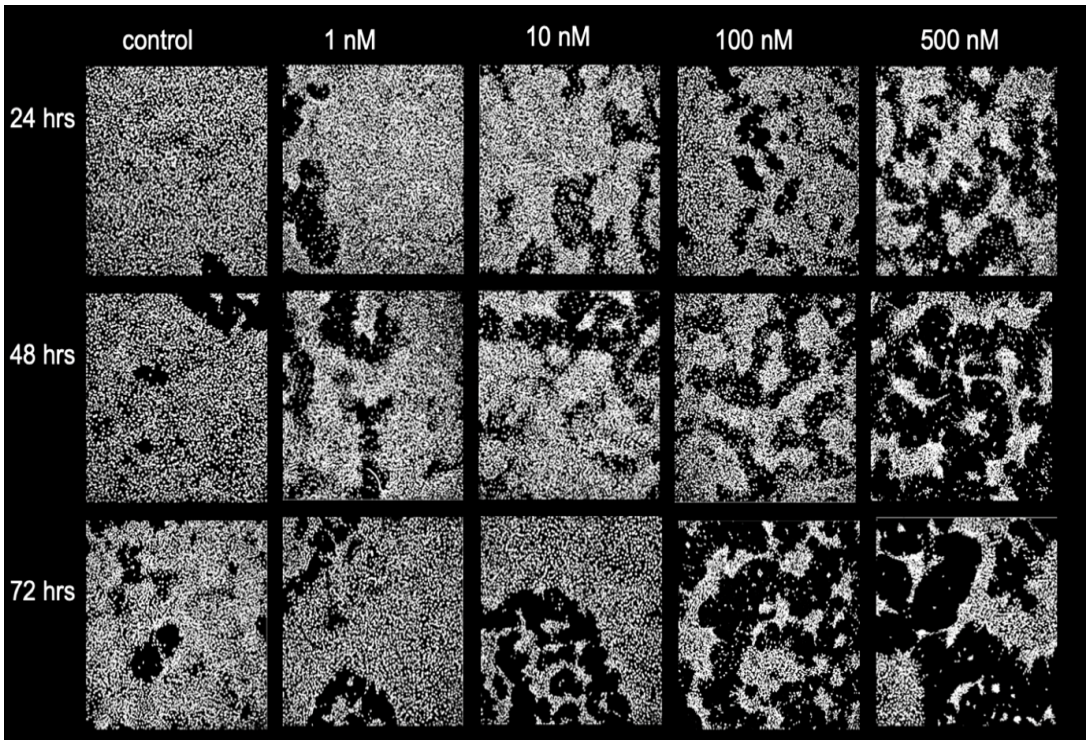

Figure 8. Representative images of control-treated and rotenone-treated 50B11 cells across 24h, 48h, and 72h time points to show the different reactions to rotenone concentrations across individual time points. Images converted to 8-bit and thresholded using ImageJ (Fiji) software.

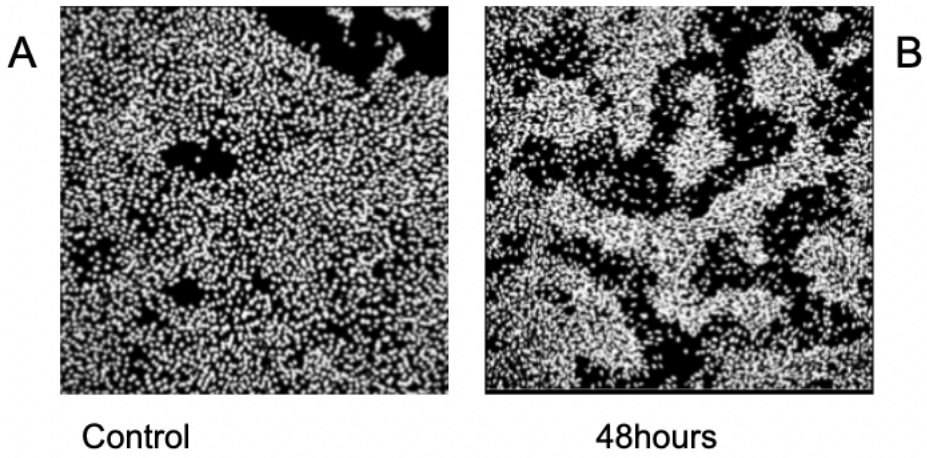

Figure 9. (A) Control-treated (DMSO) and (B) Rotenone-treated (100 nM) 50B11 cells at 48h after treatment, 10x magnification. Images converted to 8-bit and thresholded using ImageJ (Fiji) software.

Graphical Representation For Dose and Time-dependent Effect of Rotenone in 50B11 cells.

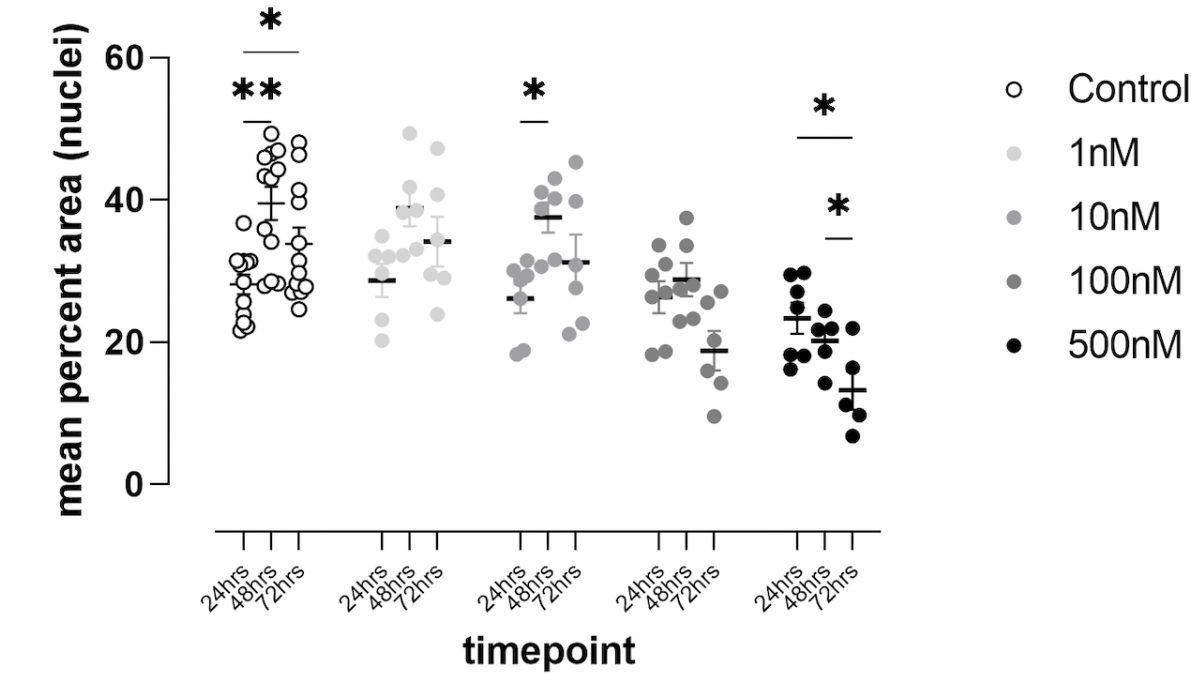

*Figure 10: Effects of rotenone concentration on 50B11cells. Analysis of mean percentage area of 50B11 cells treated with increasing concentrations of rotenone ranging from 1–500 nM at 24h, 48h, and 72h. Data are represented in separate timepoints (n = 3 experiments, percentage value analysis expressed as mean ± S.E.M: *, p < 0.05; **, p<0.05).*

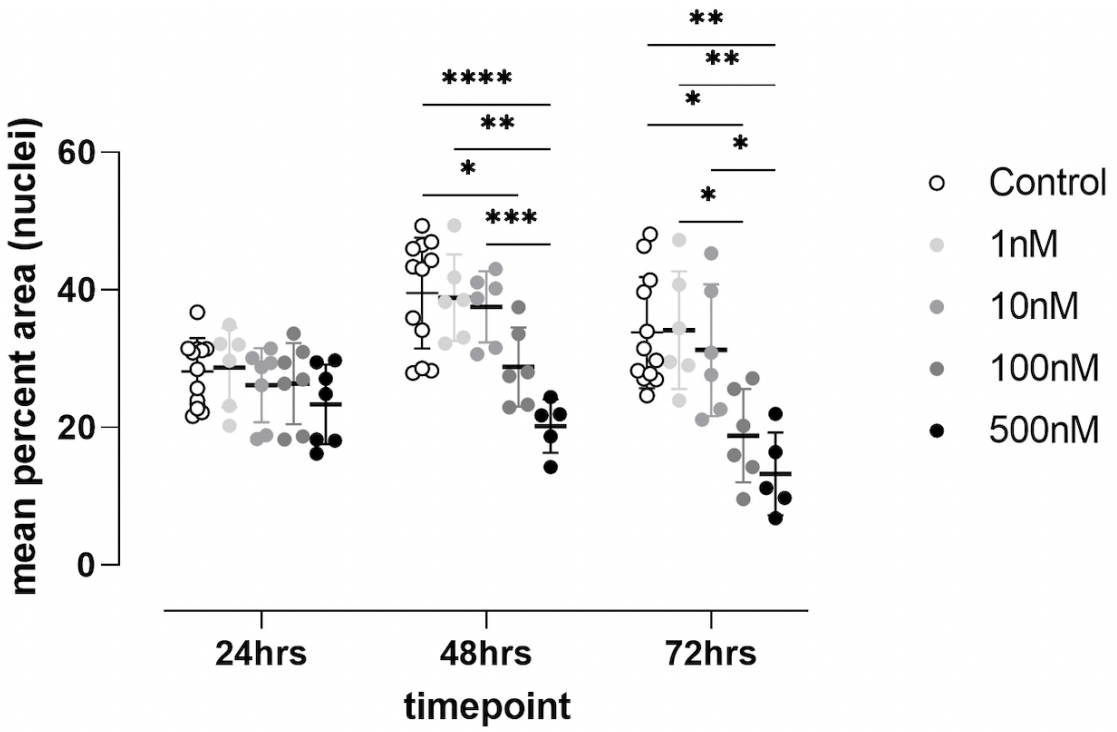

*Figure 11: Effects of rotenone concentrations on 50B11 cells. Data is same for Figure 10 but expressed per timepoint for ease of viewing dose-dependent effects. Analysis of mean percentage area of 50B11 cells treated with increasing concentrations of rotenone ranging from 1–500 nM at 24h, 48h, and 72h. Data are represented in separate timepoints (n = 3 experiments, percentage value analysis expressed as mean ± S.E.M: *, p < 0.05; **, p<0.01, ***p<0.001, ****p<0.0001).*

3.2 TMRM Imaging of cells Treated with Rotenone

The mitochondrial membrane potential (ΔΨm) generated by proton pumps (Complexes I, III, and IV) is an essential component in the process of energy storage during oxidative phosphorylation. Together with the proton gradient (ΔpH), ΔΨm forms the transmembrane potential of hydrogen ions which is harnessed to make ATP. Membrane potential is a potential gradient that forces ions to passively move in one direction. Positive ions are attracted by the negative side of the membrane and negative ions by the positive one.

Tetramethylrhodamine methyl ester perchlorate (TMRM) has been used as a sensitive probe for the detection of mitochondrial membrane potential.

A high co-transfection rate of 99% was observed in 50B11 cells when co-transfected with two different plasmids (Maili Jakobson, personal communication).

Confocal images of GFP- and channel-overexpressing 50B11 cells were obtained. Our initial observations suggest that overexpression of SCN2b reduces the mitochondrial membrane potential in 50B11 cells (Figure 12); however, further work is required to confirm this observation.

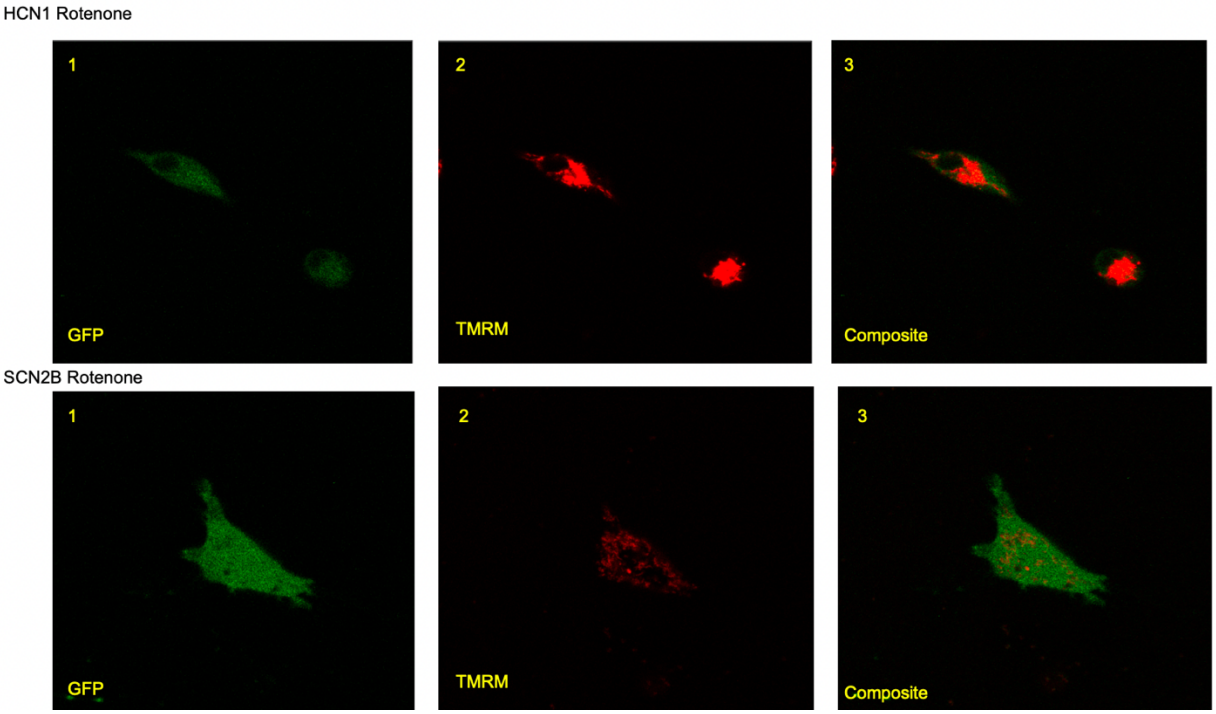

Figure 12: Representative images of 50B11 cells transfected with HCN1or SCN2B plasmids following rotenone treatment. (1) GFP (green) signal shows co-transfected cells (2) TMRM (red) stains for mitochondria (3) Composite image of cells co-transfected with GFP and HCN-1 or SCN2B with TMRM-stained mitochondria, merged using Fiji software.

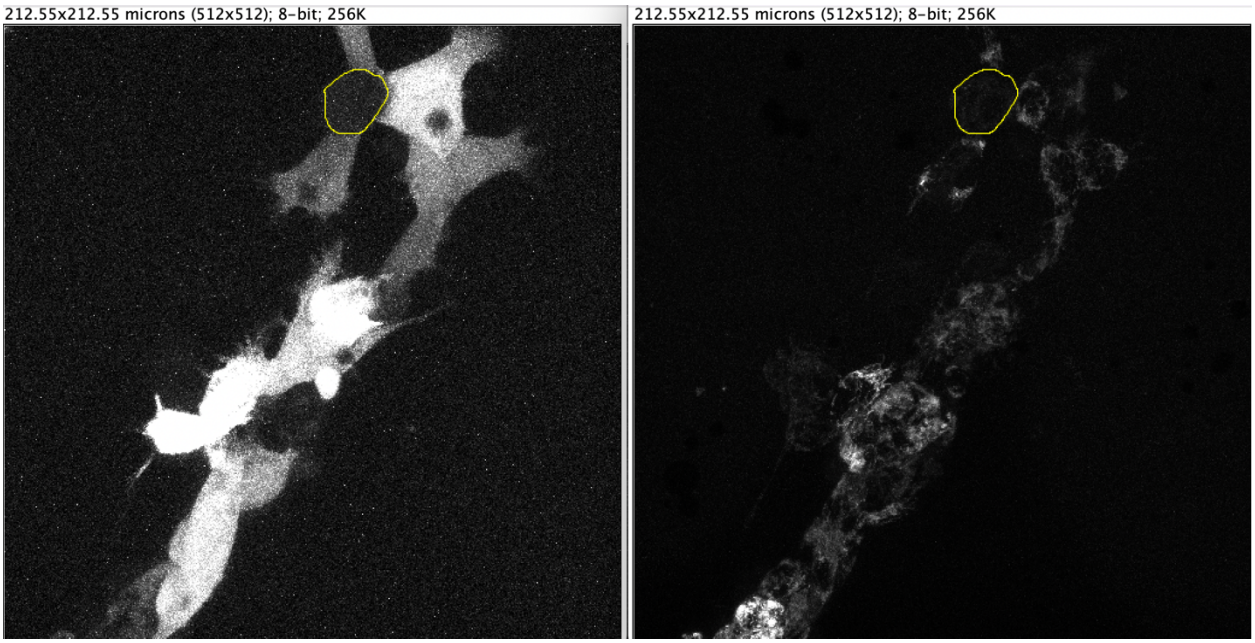

Figure 13: A 40x image from rotenone treated SCNB stained with GFP. The images here are split on image j software and the yellow contour indicates the area of untransfected cells. Contours that match both cells and mitochondria are said to be transfected while untransfected cells are measured as controls.

SUMMARY

In this thesis, I showed that rotenone affects 50B11 cells in a time and dose-dependent manner. A decrease in nuclear area fraction of up to 60% was observed when cells were treated with rotenone at a concentration of 500nM for 72h. This reduction in nuclear area (imaging was based upon nuclear staining using Hoechst) suggests that cells are highly compromised at this dosage of rotenone. Cells treated with 1nM or 10nM rotenone did not show any sensitivity to these doses and indeed, mean percentage area occupied by nuclei tended to increase over the three days, indicating that rotenone is not toxic to 50B11 cells at these concentrations.

Mean percentage area occupied by nuclei in cells treated with 100uM rotenone for 48h showed no change up to that time point, suggesting little effect of this concentration on cell survival. This also suggests that rotenone treatment at 100nM for 48h may induce subtoxic injury in 50B11 cells and hence, may be suitable to model parkinsonian (sub-acute) changes in 50B11 immortalized sensory neurons cell line.

We also imaged mitochondrial membrane potential changes in live 50B11 cells, using TMRM stain in cell cultures overexpressing SCN2B or HCN1 ion channels. Our preliminary observations show that overexpression of the SCN2B ion channel subunit may depolarize mitochondria. These observations require further analysis, which is ongoing. Future work will examine in detail the localization of these ion channels and their subunits in sensory neurons following treatment with rotenone, to better characterize the involvement of peripheral neurons in PD.

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RESOURCES

1. https://my.clevelandclinic.org/health/diseases/14737-neuropathy

2. https://www.phosphosite.org/proteinAction?id=15486&showAllSites=true

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