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Single Cell Approaches for Studying the Role of Mitochondrial DNA in Neuro-degenerative Disease

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Abstract

In light of accumulating evidence suggestive of cell type specific vulnerabilities in normal aging processes that adversely affect the brain, as well as age-related neurodegenerative disorders such as Parkinson's disease (PD), the current review highlights how mitochondrial changes could be influenced by drivers of cellular diversity. In particular, the review comments on the increased questioning of the narrow neurocentric view of such pathologies, where traditionally microglia and astrocytes have been considered players and not just bystanders in the pathological processes. Here we review the contribution made by single cell mitochondrial genome alteration towards inducing neuronal vulnerability in normal aging of the brain, and in neurodegenerative disorders, focusing on PD as a prominent example. Relatedly, we give an overview of methodologies that support such investigations. In considering the significant advances that have been made in recent times for developing mitochondria-specific therapies, investigations to account for cell type specific mitochondrial patterns and how these are altered by disease, hold promise for delivering more effective disease-modifying therapeutics.

Key words Age-related disorders, Cell specificity, Mitochondria, Mitochondrial DNA, Neurodegeneration, Parkinson's disease, Single cell analyses

1 Introduction

The last several years have seen a rapid development of technologies and methods that permit a detailed analysis of the genome and transcriptome of a single cell. This experimental approach has enabled systematic investigation of cellular heterogeneity in a wide range of tissue types and cell populations, yielding fresh insights into the composition, dynamics and regulatory mechanisms of cell states, reflective of stages of organism development but also disease.

Application of a single cell methodology is particularly important for studying the role played by defective mitochondria in human disease. Mitochondria comprise of small cytosolic organelles which evolved as a result of a symbiotic relationship that developed 1.5 billion years ago between primordial eukaryotic cells and aerobic bacteria [Martin et al., 2015]. A permanent relation was established when such bacteria developed into organelles for providing the host cells with aerobic metabolism, with the organelles that adapted to the new intracellular environment by reducing their genome size to allow for an increased replication rate.

There is mounting evidence to suggest that mitochondria are essential to the processes underlying normal aging as well as age-associated neurodegenerative disease. For aging and neurodegeneration, no known inherited mutations impacting upon the mitochondrial genome (e.g. that could encompass point-mutations, nucleotide substitutions, rearrangements, and large scale deletions, where sections of the genome sequence is missing) have been reported as causative. Instead, the mtDNA variation seen is frequently somatic and occurs over time. Although this entails that they cannot be inherited by offspring, they can be transferred to daughter cells during mitosis, or to other mitochondria during fusion and fission. As such somatic mutations accumulate in post-mitotic cells, where they impact on the

functioning of the cell [Wallace et al. 1998]. Also, being in such close proximity to the site of ROS (reactive oxygen species) generation, mtDNA can readily be oxidatively damaged, resulting in accumulation of mutations that associate with age-related decline. Since mtDNA encodes multiple components of the oxidative phosphorylation system, such mutations adversely affect this process, leading to excessive ROS generation, which in turn serves to further mutate mtDNA.

Another unique feature of mtDNA, which necessitates the need for single cell analyses, is its multicopy nature. These mtDNA copies are normally homoplasmic, meaning identical throughout the cell. However, when mutations occur, mtDNA within a single cell can be a mixture of normal and mutated copies, termed heteroplasmy. Typically, where heteroplasmy occurs the same mutated mtDNA molecules tend to occur; however, in rarer instances several copies of mutated mtDNA molecules can exist with different mutations, a concept termed pleioplasmy. When the number of mtDNA copies containing mutations within a cell exceed an arbitrary cut-off point (~60%), a functional deficit is expected to occur, manifesting principally as changes in energy production.

2 Mitochondrial DNA as the Basis of Mitochondrial Functions

Mitochondria contain their own genome (mtDNA), separate from the nuclear genome, with human mtDNA comprising of a 16,569-kb circular, double-stranded molecule. MtDNA contain 37 genes, consisting of two ribosomal RNA (rRNA) genes for protein synthesis, 22 transfer RNA (tRNA) genes, and 13 protein coding genes. These encode proteins that form part of four of the five multi-subunit protein complexes of the mitochondrial electron transport chain (ETC), namely complex I, III, IV and V, the entire complex II, as well as many other nu-

clear encoded proteins that complete the complexes. The ETC is located within the mitochondrial inner membrane. Via oxidation of the co-enzyme NADH (nicotinamide adenine dinucleotide) the ETC delivers the energy needed to synthesise ATP, thereby providing almost all of the cell's energy by means of transference of electrons to oxygen [Papa et al 2012]. However, as functional units, mitochondria not only serve to supply a cell's energy demands but also fulfill roles in apoptosis [Raule et al., 2014] and calcium regulation [Gómez-Durán et al., 2010].

The remaining ~80 subunits in the ETC are encoded by the nuclear genome [Khrapko and Turnbull, 2014]. Complex I of the ETC consists of 40 subunits, 7 of which (ND1, ND2, ND3, ND4, ND5 and ND6) are mitochondrially encoded [Signes 2018]. Unsurprisingly, considering mitochondria's ancient bacterial origins, highly conserved homologues of these genes are present in bacterial genomes [Autere et al., 2004]. mtDNA further encodes for subunits within complex III (Cytochrome B), complex IV (COXI, COXII and COXXIII) and complex V (ATPase6 and ATPase8) [Sharma et al., 2009].

Mammalian mtDNA is strikingly compact since, unlike the nuclear genome, it contains no introns and genes in some cases even partially overlap. The whole molecule is transcribed poly-cistronically before being processed to add additionally required features such as polyadenylation [Lapkouski & Hallberg, 2015]. In addition to its majority of coding regions, mtDNA also encompass two non-coding regions (NCR) sized at 700 bp in mice and 1,100 in humans. The NCR is the largest of the two at 1,124-bp and contains the promoters for transcription of both the heavy (H) and light (L) strands of mtDNA. Strikingly the NCR contains a complex structure consisting of several additional strands of nucleic acids which are found in varying abundance. The first of these to be identified was a short DNA strand

known as the 7S DNA, an approximately 0.5 kb DNA molecule within the NCR [Kasamatsu et al., 1971].

The role of the displacement (D)-loop, a triple-stranded region occurring within the main NCR of most mitochondrial genomes, is formed through stable incorporation of 7S DNA [Nicholls and Minczuk, 2014]. The function of this region is still not fully understood, although it's been shown to have important roles in mtDNA organization, segregation and replication [He et al., 2007; Nicholls and Minczuk, 2014; Antes et al., 2010], yet its abundance was found to vary greatly across cell lines and tissue types [Annex and Williams, 1990]. More recently a second molecule has been found in this region, the LC-RNA (light- (or lagging-) strand, CR RNA) that is complimentary to 7S DNA. This RNA molecule is believed important for regulating transcription of the mtDNA molecule [Reyes et al., 2020; Akman et al., 2016]. O_L is a much smaller region and contains only the origin for the replication of light strand DNA replication, with this region forming a stem loop structure when it becomes single stranded due to replication on the leading strand, and also allows replication to be initiated in the opposite direction [Fuste et al., 2010; Wanrooij et al., 2008]. Due to the essential nature of these regions for the maintenance and function of the mtDNA molecule, deletions and mutations are usually significantly reduced in frequency in these regions.

mtDNA molecules exist in multiple copies per cell varying between as much 10^3-10^4 copies per cell (mtDNA copy number (CN)), depending on the particular cell's bioenergetic demands. As mtDNA replicates independently of the cell cycle this number can change in relation to nuclear DNA levels dependent on the cells proliferation rate but is also regulated by mitochondrial specific processes such as mitophagy and levels and functionality of mitochondrial proteins such as TFAM (transcription factor A, mitochondrial) and POLG (polymer-

ase γ). In healthy adult brains, the number of mtDNA molecules, or mtDNA CN, varies between different cell types. mtDNA CN is relatively high in neurons, due to the exceedingly high energy demand posed by the brain. Thus, analysis of mtDNA at a single cell level allows us to distinguish how levels change between different cell types , with age and activity etc, rather than looking at an average across tissue, which may well not give us an accurate view of deeper variation within the sample.

Although research at a single cell level has shown that mutations in mtDNA often differ between individual cells, mtDNA molecules within a single cell commonly contain the same point mutation [Khrapko and Turnbull, 2014]. This supports the theory that a mutated molecule can undergo clonal expansion to outnumber their wild-type (wt) mtDNA counterparts and become the dominant allele within a cell. It is still unclear whether mutant mtDNA molecules can proliferate due to some select advantage or by random chance. However once mutated mtDNA molecules reach a threshold of 60-90%, a biochemical defect is usually detectable including reduced activity of cytochrome c oxidase (COX) [Grady et al., 2014]. However, with several thousands of copies of mtDNA in individual neurons, mutant molecules existing at very low frequencies, are viewed as recessive and only produce detectable effects when the majority of the mtDNA molecule population within a specified cell contain the same mutation [Khrapko and Turnbull, 2014]. Again single cell analysis gives additional vital understanding to the distribution of mtDNA mutations and their impact. For example when looking at a population of cells a mutation may appear to be at a low level within the population, but it is not clear if that mutation is found at a low level across all cells or may be at a high abundance in a much smaller number of cells, however, two such different populations could lead to significant different impacts and arise in very different ways.

3 Susceptibility of mtDNA in Parkinson's disease: A Growing Realization for Cell-Type Specific Variation

A case in point for application of methodologies for assessing the extent of mtDNA variation and its biochemical and physiological consequences relates to investigations concerning Parkinson's disease (PD), a progressive neurodegenerative disease that transcends into a manifestation of both motor and non-motor symptoms [Moustafa et al., 2016; Balestrino et al., 2017]. It is becoming increasingly recognised that non-motor features of the disease can be significantly more debilitating and resistant to treatments than the motor aspects, which include rigidity, tremor at rest and bradykinesia. PD-related non-motor symptoms are diverse and include dementia, mild cognitive impairment, psychosis, apathy, restlessness (akathisia) and impulse control disorders as well as depression, anxiety, sleep disorders, orthostatic hypotension, constipation and pain. Such symptoms can be localized to the cortex, basal ganglia, brainstem, spinal cord and peripheral nervous system. It is now well established that the cardinal motor symptoms of PD are due to the death of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc), a midbrain dopaminergic nucleus that forms part of the basal ganglia circuitry, and which fulfill a critical role in modulating motor movement and reward functions [Alexander, 2004]. A further core neuropathological characteristic of PD brains is the presence of Lewy bodies (LBs) and Lewy neurites (LNs) containing abnormal misfolded protein aggregates, including α-synuclein (α-SYN) that accumulate intracellularly [Trojanowski et al., 1998] and has been proposed to progress through the brain caudo-rostrally [Braak et al., 2003]. In this regard, accumulating convincing evidence suggests for the prion-like qualities of the α-SYN protein due to the neuron-to-neuron transfer that has bene noted in grafted neurons transplanted in PD patient brains over a decade prior to autopsy

[Hansen et al., 2011], in cell culture [Desplats et al., 2009; Luk et al., 2009] and also in experimental animal models [Luk et al., 2012]. In PD patients, LB progression throughout the brain has also been correlated with disease severity [Braak et al., 2003]. However, whether these intracellular inclusions comprise a pathological substrate of the clinical syndrome of PD, or whether their presence rather indicates neuroprotection, remains to be determined. Regardless of this ongoing debate, the notion of PD being regarded as a synucleinopathy now seems firmly established.

Over recent years it has become increasingly apparent that degeneration of neurons and the formation of abnormal protein aggregates within the remaining neurons of PD patient brains are not limited to the SNpc dopaminergic neurons, thereby sparking significant interest as to the degree of involvement of cholinergic, serotoninergic, noradrenergic and other neurotransmitter systems, which provide the neural substrate for several PD-associated symptoms. In this regard, evidence was given that dysfunction and/or degeneration of regions such as the locus coeruleus (LC) and dorsal raphe nucleus, the main sources, respectively, of norepinephrine and serotonin (5-hydroxytryptamine; 5-HT) in the brain, contribute especially to 'axial' signs and cognitive impairment. Another brain region and neuronal type that has gained prominence as being likely to be a major cause of disability during disease evolution in PD are the large 'Ch5' cholinergic neurons of a brainstem nucleus, the pedunculopontine nucleus (PPN) [Mesulam et al. 1983; Rinne et al. 2008; Pienaar et al. 2013; Bury et al. 2017]. Although other cell types, including glutamatergic, GABAergic and glycinergic neurons that may co-express a variety of neuropeptides, also locating to this heterogeneous nucleus may also be lost during PD progression [Standaert et al. 1986; Austin et al. 1995; Mineff et al. 1998; Wang & Morales 2009; Martinez-Gonzalez et al. 2012; Pienaar & van de

Berg 2013; D'Onofrio et al. 2015], the resident cholinergic population remains the most severely affected [Pienaar et al. 2013], where a PPN cholinergic lesion induces dysfunctions in the complex neural network that the PPN forms a key component of.

Reasons that render the SNpc dopaminergic and PPN cholinergic neurons particularly vulnerable to PD disease processes remain to be fully determined; however, striking similarities deemed important contributing factors are noted between these two structures, including the very long neuronal projections and extensive arborisations inherent to both SNpc dopaminergic and PPN cholinergic neurons. In each case, a far less dense portion containing other types of neurons is also present, where such neurons contain a far more restricted projection profile compared to the adversely impacted dopaminergic and cholinergic neurons [Gutt & Winn 2016].

Other inherent factors that may explain (or at least contribute) to the selective vulnerability of certain cell populations include dysfunctional brain iron metabolism, particularly affecting the uptake, storage and release of iron [Jiang et al. 2019]. In particular, iron-related mechanisms have been deemed potentially important for the large-scale cell death affecting nigral dopaminergic neurons as well as the tendency for remaining ones to undergo α-SYN aggregation, lending support to the use of iron chelator drugs as a plausable therapeutic strategy for PD [Lewis et al. 2020]. A plausible mechanism by which nigral dopaminergic neurons, in particular, are susceptible to excessive iron deposition may relate to interaction of iron with neuromelanin, a dark coloured granular pigment present in this neuronal group [Mochizuki et al 2020]. Shamoto-Nagai and others [2006] provided some details as to this interaction by showing that iron released from neuromelanin increases oxidative stress within the mitochondria, thereby causing mitochondrial dysfunction and, in turn, reduce proteasome function. Whether altered iron levels associate with loss of other cells that lie

outside the SNpc remains largely unexplored, with only the LC that has been explored in this regard, showing no such association [Zecca et al 2004; Zucca et al 2006].

The diversity of cell types affected during PD pathogenesis go far to explain the wide range of symptoms displayed by PD patients, while providing multiple targets for pharmacological interventions to treat such symptoms. Brain vasculature is another non-neuronal cell type that may also be affected during PD. For instance, our group reported extensive damage affecting microvessels within the subthalamic nucleus (STN), as was assessed in post-mortem PD brains [Pienaar et al. 2015]. In particular, we found striking downregulation of vascular adherens junction, tight junction-associated proteins and also vascular endothelial growth factor (VEGF) in the PD-afflicted brains compared to neurological controls, as well as several adverse structural alterations in the PD samples, including severe thinning of microvessel endothelial cell thickness and also shortening of such vessels. Interestingly, this research report also demonstrated that a surgical therapeutic intervention termed deep-brain stimulation (DBS) greatly improved these cellular and molecular aspects in PD patients when compared to patients that did not receive STN-DBS.

Astrocytes, comprising the most populous glial subtype is a brain residing cell type that has received significantly less research attention with regards to a possible role in PD [Booth et al. 2017]. As critical regulators of a variety of brain functions, they provide structural and metabolic support, and also regulate synaptic transmission, water transportation and blood flow within the brain [Sofroniew & Vinters, 2010]. Recent evidence indicated that disrupted astrocyte biology might have a fundamental role in neuronal degeneration seen in the brains of PD patients. In this regard, monogenetic mutations affecting 17 genes that have been implicated in the development of the disease were found expressed in the astrocytes

of different human and mouse brain cell subtypes, in some instances higher than in neurons [Zhang et al. 2016].

Moreover, evidence suggesting an immune response in the brains of PD patients has increased greatly, implicating microglia cells, which play a major role in the inflammatory process by rapidly responding in a phagocytic manner to pathological insults [Streit et al. 2004; Gelders et al. 2018]. Recently, George and others [2019] reported that microglia, when activated due to pro-inflammatory stimuli, increasingly partake in α-syn cell-to-cell transfer, thereby promoting prion-like spread of the pathology. Such results suggest that modulation of the innate immune system might mitigate formation and accumulation of intracellular misfolded protein aggregates, which characterizes the brains of PD patients.

PD has a multifactorial etiology, with a variety of genes and molecular pathways that may contribute to the development and progression of this disease, including those involving mitochondria, the organelle providing the majority of cellular energy via adenosine triphosphate (ATP) production. As neurons require high energy levels to maintain intracellular ion concentrations, often against membrane concentration gradients, an impairment to mitochondrial-derived energy could lead to neuronal dysfunction [Fachal et al., 2015] and even cell death. The link between mitochondrial dysfunction and PD was first investigated in the 1970s when MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a prodrug to the dopaminergic-targeting neurotoxin MPP+ (1-methyl-4-phenylpyridinium), was found to induce a condition resembling idiopathic PD in drug users [Ballard et al., 1985]. A variety of toxic mechanisms has since been proposed to explain the killing of dopaminergic-specific neurons [Choi et al., 2015]. However, the main mechanism involves inhibiting mitochondrial complex

I, present within the inner mitochondrial membrane [Nicklas et al., 1985]. Complex I comprises the first component of the ETC, responsible for generating ATP via a series of redox reactions for oxidative phosphorylation (OXPHOS).

A strong case for mitochondrial damage in PD pathogenesis derives from an extensive body of literature using neurotoxin animal models of PD, where animals reflect core clinical and post-mortem neuropathological features of clinical PD to a remarkably accurate extent. Previously the dopamine transporter (DAT) was deemed to play a crucial role in the dopaminergic-specific toxicity of MPP+ [Javitch et al*.,* 1985; Gainetdinov et al*.,* 1997]. However, although it's been known for some time that MPP+ also acts as a high affinity substrate for the serotonin transporter, which helps explain the MPP+-induced damage sustained by the serotonergic neurons, recent findings reported by Martí and others [2017] found that MPP+'s toxic mechanism-of-action differs between these two monoaminergic neuronal types, particularly relating to differential effects on neurotransmitter release and re-uptake. Previous human post-mortem PD tissue analyses revealed depletion of 5-HT levels within serotonergic neurons that originate from the raphe nuclei [Braak et al., 2003]. The additional finding of significant variance of 5-HT levels across patients, with the concentrations of some PD patients that fell within the range of the healthy controls [Shannak et al., 1994; Kish et al., 2008] suggests for differential susceptibility towards PD-related pathological processes between these two neuronal types. The mechanism by which MPP+/MPTP induces neuronal death is through mitochondrial insult, particularly via inhibition of complex 1 of the ETC. Thus, this environmental toxin is regarded as a Parkinsonian mimetic, as a similar dysfunction is noted in nigral dopaminergic neurons of sporadic PD cases [Schapira et al., 1990]. Although MPP+ is a high affinity substrate for both DAT, rendering dopaminergic

neurons susceptible to MPP+'s neurotoxic effects, the toxin also has affinity (although comparatively lower) for the serotonin transporter, meaning that serotonergic neurons are also vulnerable to MPP+-induced neurotoxicity [Martí et al., 2017]. However, the vast bulk of MPP+ PD modelling experiments have been performed on dopaminergic-type neurons, leaving the question of whether 5-HT neurons might be less susceptible to MPP+ toxicity, and the mechanisms that might underlie such neuroprotection, largely unanswered. The study by Martí and colleagues [2017] enlightened on possible differences to explain the differential susceptibilities by treating stem cell-derived dopaminergic and serotonergic neurons *in vitro*. Whereas MPP+ exposure impaired mitochondrial membrane potential in both cell types, only dopaminergic neurons were affected in terms of 'synaptic vesicle cycling', a molecular process entailing constant recycling of synaptic vesicle protein complexes for effective neurotransmitter release. This result, along with the finding of contrasting differences in MPP+-induced trafficking of neurotransmitter transporter proteins between the two monoaminergic neuronal types indicates that mitochondria metabolism is similarly affected by the toxin treatment, but that the PD-relevant mitochondrial toxin MPP+ exerts different effects on neurotransmitter release and re-uptake, depending on the neuronal type.

Another neurotoxin utilized in PD research, for elucidating on neuropathological mechanisms that converge to adversely affect mitochondrial functions in neurons selectively vulnerable to PD, is the piscicide, rotenone. By also blocking complex I of the ETC, rotenone exposure results in a loss of ATP synthesis, in a manner similar to MPP+ [Krueger et al., 1990], although later work revealed that bioenergetic defects induced by rotenone exposure are not responsible for cell death [Sherer et al. 2003], whilst also producing superoxide anions [Hasegawa et al., 1990]. Characterisation of the rotenone rat model of PD substantiated involvement of pesticide exposure and systemic complex I dysfunction in PD aetiology, with

rotenone-treated rats that feature several motor symptoms of PD such as resting tremor and muscle rigidity, as well as cellular pathology overlap with clinical PD, including selective nigrostriatal dopaminergic degeneration and α-SYN-positive cytoplasmic inclusions [Betarbet et al., 2000]. The MPTP mouse and rotenone rat models are examples of relevant preclinical animal models that reproduce PD pathology with high fidelity. The fact that pathological processes converging on mitochondrial functions are identified consistently as critical mechanisms underlying the pathological profile these animals present with, provide firm evidence that altered mitochondria are key players in progressive PD.

Extensive additional evidence for defective mitochondria's role in PD pathogenesis derives from the consistent link seen between oxidative stress and monogenically inherited PD-associated genes. Whilst most cases of PD (~90%) are sporadic, of the nuclear genes identified to play a causative role in familial forms of PD, several are known to encode mitochondrial proteins. These proteins form components involved in pathways that are vital for balancing mitochondrial numbers through biogenesis and mitophagy. Mutations to the nuclear genes *PINK1, DJ* and *PARK2* can also directly compromise the rate of OXPHOS (Hudson *et al.*, 2013). Several genes linked to PD have also been implicated in mechanisms underlying mitochondrial response to stress and autophagy [Pyle et al., 2016). Mitochondrial dysfunction has also been observed at the early stages of PD progression [Pyle et al., 2016] with defects in mitochondrial function causing impaired respiratory chain complex I in SNpc dopaminergic neurons [Hudson et al., 2013]. This has led to the proposal of mitochondrial dysfunction and, consequently oxidative stress, through increased generation of ROS, as a contributory factor in the etiology of PD.

In the sections above we highlighted work showing that mitochondrial dysfunction and oxidative stress associates with neuronal loss that characterizes PD-affected brains

[Schapira, 2008], how inhibitors of complex I of the mitochondrial ETC results in parkinsonism [Betarbet et al. 2000], and the multitude of studies which reported the presence of a deficient mitochondrial ETC complex I in PD patients [Schapira et al., 1989; Parker et al., 1989; Mizuno et al., 1989]. Whilst haplogroup studies have been useful for investigating inherited mutations in mtDNA as a driver of PD, these studies are unable to detect the impact of somatic mtDNA mutations that accumulate over time in individual cells. This illustrates the importance of investigating somatic mtDNA mutations at a single cell level, as individual cells will acquire different mutations and levels of mtDNA deletions will vary between cells. However, in contrast to inherited mutations, where all mtDNA molecules in a cell contain the same mutation, somatic mutations occur at different frequencies and not all mtDNA molecules are likely to contain the same mutation. As the mitochondrial genome is present in higher numbers and undergoes greater numbers of division than the nuclear genome, the mitochondrial genome is more susceptible to acquiring mutations over time in both tissues from normal aged individuals, and those from diseased cohorts. MtDNA is more susceptible to oxidative damage and is prone to a higher mutation rate than the nuclear genome, due to the lack of histone-like packaging proteins and the location of mtDNA in close proximity to the site of ROS production, thereby rendering mtDNA more susceptible to mutations over time than nuclear DNA, with concomitant oxidative damage. MtDNA single nucleotide polymorphisms, -deletions, -insertions and CN changes associate with susceptibility to and progression of neurodegenerative disease, including PD [Coppede and Migliore, 2015]. This has been extensively described in one neuronal population that is vulnerable to degeneration in PD, namely the dopaminergic neurons whose cell bodies exist in the SNpc. For this, Swerdlow and others [1996] reported that this complex I defect can be transferred to cell lines by expressing mtDNA taken from PD patients, implicating somatically acquired lesions

affecting the mitochondrial genome in PD pathogenesis. Additional evidence in support of this relates to findings that the SNpc of post-mortem PD patients harbour substantial levels of the types of Δ-mtDNAs frequently seen in aged brains also [Kraytsberg et al. 2006; Yao and Wood, 2009].

Increased large-scale mtDNA deletions have been reported present in post-mortem PD-affected dopaminergic neurons of the SNpc [Bender et al. 2006; Lin et al. 2012; Coxhead et al. 2015; Dölle et al. 2016]. Such neurons also displayed reduced mtDNA copy number [Pyle et al. 2015; Dölle et al. 2016], thus reducing the pool of wt mtDNA, which would normally increase with age as compensation for accrued damage over time [Müller-Nedebock et al. 2019]. In all such studies it is important to consider age matched controls in these studies as ageing is the biggest risk factor for PD, with post-mitotic neurons inherently vulnerable to age related decline. The findings from such carefully controlled post-mortem studies have led to the hypothesis that accumulation of such somatically produced Δ-mtDNAs may eventually cause a bioenergetic deficit, thereby contributing to PD-related neurodegeneration, with this postulation that received indirect support from studies done on mice harboring a mtDNA deficiency within the nigral neurons, where the animals showed a parkinsonian phenotype [Ekstrand et al 2007].

Ongoing efforts by our lab and others, to identify the nature and spectrum of mtDNA changes affecting different cell types as a consequence of PD pathology, could facilitate the discovery of earlier pathophysiological markers, along with more targeted therapeutic strategies. Studies on post-mortem human brain tissue have shown reduced mtDNA CN in SNpc dopaminergic neurons [Pyle et al., 2016]. Interestingly, this study found no association between haplogroup and mtDNA CN [Pyle et al., 2016]. Another study reported that mtDNA CN did not significantly differ between SNpc neurons in PD brains with LBs compared to

those without LBs [Müller et al., 2013]. In contrast, cholinergic PPN neurons show elevated mtDNA CN [Bury et al., 2017]. Interestingly studies on both nigral dopaminergic and brainstem cholinergic neurons demonstrated increased rates of deletions in mtDNA in PD compared to healthy neurological controls.

As neurons, particularly excitatory neurons such as dopaminergic and also cholinergic ones, require high levels of energy to allow ions to pass through their cellular membranes, any defects in energy production could result in impaired neuronal function, manifesting as disease symptoms [Fachal et al., 2015]. SNpc dopaminergic neurons are particularly prone to mitochondrial dysfunction, which could take the form of reduced mitochondrial ETC protein expression and related activity [Schapira et al., 1990], and/or ROS-mediated damage. However, few studies have been done to date for determining the extent and nature of mitochondrial dysfunction in other, non-dopaminergic cell types implicated in PD pathogenesis. This is an area of research that should be expanded upon to help in designing cell-specific strategies for combatting PD.

A reduction in mtDNA CN could indicate errors in mtDNA replication or increased mitophagy at a cellular level. Conversely an increase in mtDNA copy number could serve as a response to maintain wt mtDNA populations and rescue mitochondrial function within cholinergic neurons [Bury et al., 2017]. Differences may also entail involvement of different nuclear genes, where large deletions or point-mutations occuring in the mtDNA can be the result of defects in these genes, which are involved in replication of the mitochondrial genome [Khrapko and Turnbull, 2014]. However, the presence of different mtDNA mutations between individual cells suggests that some of these mutations arise somatically, independent of the nuclear genome. In addition, metabolic changes may also be a contributing factor in the accumulation of mtDNA deletions. For instance, metabolism of catecholamine, a key

synapse-based communication currency for several neuronal types (including dopaminergic neurons), is altered as a result of PD pathology. In this regard, recent *in vitro* and *in vivo* studies, Neuhaus and colleagues [2014] provided experimental data to propose that altered catecholamine metabolism due to PD neuropathological processes, could drive the accumulation of mtDNA deletions observed in SNpc dopaminergic neurons of aged brains, with this phenomenon that is exaggerated in PD. In one experiment, the investigators showed in mouse brains, a preference for mtDNA deletions to accumulate within dopaminergic regions, namely the SN, striatum and cortex, compared to the cerebellum, a brain region void of dopaminergic somas or terminals. The results concluded that brain regions residing high dopamine metabolism preferentially accumulate mtDNA deletions. Whether a similar principle might underlie the preferential accumulation of mtDNA deletions in cholinergic neurons, and if such a phenomenom varies across brain regions, will require similar comparative analysis but on cholinergic neurons, producing the neurotransmitter acetylcholine (ACh), to other types relying on different neurotransmitters.

Studies to explain the mechanistic basis for differences in the pattern of mtDNA variation between various cell types in PD-affected brains promise to help identify therapeutic targets to exploit in pharmacological screens. In this regard, an accumulating body of work suggests the neuroprotective potential of such therapeutic strategies to influence the course of neurodegenerative disease, as was recently reviewed [Weissig et al., 2020]. Examples of mitochondria-targeted therapeutics for alleviating or even preventing accumulation of mtDNA defects include antioxidant therapy, pharmacological modulators of mitochondrial dynamics or of mitochondrial epigenetics.

4 Experimental Methods for Exploring Cell-Specific Brain Pathology

Brain tissue contains different types of neurons and supporting cells, with each type of cell having different functions and molecular compositions. Methods for precisely isolating cells from the surrounding tissue, promise to uncover the spectrum of cell types in the brain and their individual molecular profiles that would otherwise be obscured by whole-tissue approaches. Cell sorting techniques for distinguishing between different cells within tissue can be undertaken in several ways, many of which are becoming cheaper, easier and faster. Traditional techniques include fluorescence-activated cell sorting and laser capture microdissection, while techniques which allow for improved study of the genome, epigenome and transcriptome at single cell resolution are also being developed.

Application of single cell techniques to postmortem tissues can pose significant technical challenges, due to the age or fragility of samples. For example, tissue samples may have been frozen for many years and therefore ways need to be devised to stabilize the cellular membrane for cell sorting. However, significant progress has been made in this area, and there is now a wealth of published methods and a number of specialist labs developing these to be accessible for all.

5 Methods

5.1 Tissue Preparation, Positive Identification of Cell Type and Cell Isolation for Downstream Applications

Non-fixed, frozen brain blocks containing the region of interest (ROI) should be cut at a thickness optimal for the cell isolator system to be applied to effectively. Sections should be placed on slides of the type best suited for the type of cell isolation apparatus to be used. For instance, laser capture microdissection (LCM) systems comprise of either a “drop-down”

or a “catapult” design, where the drop-down version requires slides where tissue sections are placed on a thin plastic membrane, while the catapult version entails placing the sections on non-adhesive glass slides for the cell’s easy removal from the surrounding tissue. For downstream molecular applications involving analysis of DNA or RNA, every effort should be made during the tissue handling process to prevent the degrading influences of RNAases and DNAases, i.e. by spraying the inside of the cryostat chamber with commercial products such as RNaseZAP®, formulated to mitigate the harmful effects caused by such nucleases that catalyse the degradation of RNA or DNA into smaller components.

From serially-cut brain tissue sections, various histochemistry protocols, designed to allow for clear visualization of neuronal populations and white matter fiber tracks, to aid identification of the ROI, can be used on the first and last sections in the series in order to confirm presence of the ROI in-between. Typically such stains include:

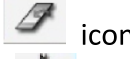
- Luxol Fast Blue (LFB) staining, for visualising myelin and therefore the white fibre tracts (Fig. 1A).
- Haematoxylin & Eosin (H&E) staining. Haematoxylin stains the nuclei blue, whilst eosin stains the extracellular matrix and cytoplasm pink (Fig. 1B).

Once its confirmed that the sectioned tissue contains the required ROI, an antibody-based “quick-stain” is applied to the tissue sections to visualize the cell type to isolate from the tissue using cell capture technology. The procedure entails:

- Take glass slide-mounted tissue sections out of the -80˚C freezer and air-dry them in a class II cabinet for 30 minutes
- Ensure that all condensation has evaporated before continuing
- Lightly wet a layer of paper towel and place inside a staining tray
- Once the tissue sections are dry, trace around each section with a hydrophobic marker pen
- Rinse each section with TBST (a mixture of tris-buffered saline (TBS) and Polysorbate 20, also known as Tween 20) for 3 x 5 minutes
- Tip off the excess TBST and add pre-vortexed 5% blocking serum (horse) in TBST (1% Tween) for 30 minutes at room temperature (RT)

- After the blocking step, tip off excess blocking serum and add pre-vortexed and centrifuged primary antibody at an optimal concentration, with the antibody diluted in TBST; incubate for 2 hours at RT in the class II cabinet
- Following this incubation period, tip off the excess primary antibody solution and add the solution containing the species-specific horseradish peroxidase (HRP)-conjugated secondary antibody at a concentration of 1:200, diluted in TBS and incubate for 1 hour at RT
- Tip off the excess secondary antibody solution and then wash the sections in phosphate buffered saline (PBS) for 3 x 5 minutes
- Remove the excess PBS and then apply the chromogenic substrate TMB (3,3′,5,5′-Tetramethylbenzidine) for 10 minutes at RT; note that TMB is light sensitive so light exposure should be minimised
- Wash sections briefly with nuclease-free water
- Use immediately for single cell isolation

From such stained sections, the cells of interest are then captured using a cell capture system, such as an LCM device. This involves using a laser to cut around a cell and then using a catapult system to collect the cell into an Eppendorf tube (Fig. 2). When using the P.A.L.M. MicroBeam Laser-Capture Microdissection system coupled to an inverted Zeiss microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany), the procedure involves:

- Tissue sections should be cut at 20μm thick or less to allow laser-assisted microdissection to work optimally
- Apply the “quick-stain” methodology (see above) to visualize the cell population of interest
- Turn on the equipment in the following order: Mains (extension lead), computer and then the power supply of the LCM; select “Zeiss” programme from desktop
- To insert a tissue section slide click the  icon
- To navigate to a cell click on the compass icon
- Insert a 0.2ml Eppendorf tube into the tube collector with the cap facing down
- Select the “autocut” option, or select “Settings” à “Preferences” à “Laser” à Select and subsequently adjust the minimum diameter of the laser beam of the LCM microscope (between 7.5 μm and 30 μm), depending on size of cell (20 μm is normally required for larger neurons)
- To test settings, draw a large random spiral in an area of the tissue lying outside the ROI; adjust the settings to obtain a smooth cut with the laser set at the minimum possible “energy cut” setting to avoid scorching intended cells
- Click icon to access “cap move” interface; select “cap 1” and then click the for drawing around the cell; ensure you draw closely around each cell but leave a slight margin to ensure that the cell itself is not damaged by the laser-evoked energy

- Click the icon to start the laser cutting
- Once the first cutting has finished click “cap 2” on the “cap move” interface and then draw around the second cell to repeat the process
- To remove the tube from the device click the icon
- Centrifuge the collected cells for 10 mins at 12,000 rotations per minute
- Place the tubes on ice and commence with molecular analytical methods immediately

This procedure described use of an LCM device that catapults the target cell into the adhesive cap of a microfuge tube. An alternative system on offer rather drops the specimen into the tube using gravity. In this case, sections are placed on specialized plastic membrane slides, where the membrane acts as a stabilizing scaffold.

Although no systematic studies have been performed so far to conclusively demonstrate this, concern exists that LCM-induced scorching may induce false mutations in samples. However, the most prominent concern relating to the use of LCM is the cost for acquiring such a devise that places single cell studies out of reach for most labs. A more economical option for cell capture from tissue, which also overcomes the issue relating to inadvertent laser-evoked scorching of the cell’s borders, was recently introduced, known as a “UnipicK”™ apparatus (Fig. 3). This system uses the cutting edge of a glass capillary tube to cut out the cell of interest from the surrounding tissue before vacuum suction is applied to capture the cell into the same glass capillary tube, from where it can be flushed into a microfuge tube. In our hands, we have found that the system allows more precise control over an LCM apparatus for obtaining a specific cell, thereby avoiding contaminating the sample with unwanted material such as surrounding glial cells. However, the system can only reliably cut through tissue sections of 15µm thick or less, which could be problematic if working on rare specimens provided by a collaborating lab (hence the receiving lab had no control over preparing the sections for subsequent cell isolation studies). Furthermore, we have

also found that the tissue section needs to be constantly hydrated with sterile buffer containing a very small amount of detergent (e.g. Tween-20) for its cutting function to work effectively. In this regard, care should be taken to remove this bathing solution from the sample (i.e. through centrifugation) before commencing with downstream molecular analyses.

5.2 Mitochondrial Copy Number Variation and Measurement of mtDNA Deletion Levels

Mitochondrial copy number (mtDNA CN) refers to the number of copies of the mitochondrial genome present in a cell. mtDNA CN is typically higher in active cells such as neurons and muscle cells, but also varies on an intercellular basis [Guyatt et al. 2019]. Measuring mtDNA CN is a useful tool for identifying changes in rates of mitochondrial biogenesis and mitophagy in PD.

A commonly used technique to measure mtDNA CN is quantitative real-time PCR (qPCR). In this technique, quantifying expression levels of a stable mitochondrial gene *mtND1* against *mtDN4*, a mitochondrial gene that is deleted in >95% of mutated mtDNA molecules [Grady et al. 2014] can be used to measure the number of wild type molecules and molecules harbouring deletions to give an estimation of heteroplasmy levels. Alternatively, the expression levels of *mtND1* can be measured alongside the levels of a nuclear housekeeping gene such as *B2M* to determine mitochondrial CN per cell. Below we provide a stepwise methodology for single neuron (post-mortem brain) mtDNA CN analysis, as previously published in our paper [Bury et al. 2017].

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Gross changes in mtDNA can by observed through microscopy and imaging [Legros et al., 2004, Kukat et al., 2011] but to get more quantititative and higher throughput measure a commonly used technique to measure mtDNA CN is quantitative real-time PCR (qPCR) (hunter 2010). This technique allows several pieces of information to be gained from each cell with deletion levels and total copies relative to nuclear DNA to be assayed together in a multiplex reaction [Grady et al. 2014]. Where a multi- cell population is used nuclear DNA must be quantified using levels of a nuclear housekeeping gene such as *B2M* to determine mitochondrial CN per cell. However where a single cell can be assured per reaction this is not necessary. By utilising primer and probe sets localized in different regions of the mtDNA data can be obtained about the number of molecules per cell which are incomplete (negative for a particular region). A large proportion (>95%) of deleted mtDNA molecules are missing a region between O_H and O_L, termed the “common deletion” [Reeve et al. 2008; Damas et al. 2014; Belmonte et al. 2016]. Due to their strategic location it is believed that these are generated during replication [Tuppen et al., 2010]. Thus by selecting two sets of primers and probe sets, *mtDN4* in the deleted region and *mtND1* on the other side of the circle as a control, relative total copy number and deletion heteroplasmy levels can be calculated for multiple single cells.

Below we provide a stepwise methodology for single neuron (post-mortem brain) mtDNA CN analysis, as previously published [He et al., 2002; Pyle et al., 2016].

Reagents:

- mtND1 forward primer sequence: ACGCCATAAAACTCTTCACCAAG
- mtND1 reverse primer sequence: GGGTTCATAGTAGAAGAGCGATGG
- mtND1 probe Hex-: CCCGCCACATCTACCATCACCCTC- BHQ-1
- mtND4 primer Fwd: ACCTTGGCTATCATCACCCGAT
- mtND4 primer Rev: AGTGCGATGAGTAGGGGAAGG
- mtND4 Probe CY5-CAACCAGCCAGAACGCCTGAACGCA-BHQ-2
- The qPCR mastermix: A range of these are available, e.g. Luna® Universal Probe qPCR Master Mix (NEB) –ensure selected one is compatible with the qPCR machine to be used Tris HCl, pH 8 (Fisher Scientific)
- Tween-20
- Proteinase K
- Cells, suitable plasmid/PCR products to make standard curve
- 96-well plates
- Plate seals suitable for the q-RT-PCR machine available

Method:

- Isolate single cells from the tissue as described above
- Place each cell into a PCR tube with 35µl of lysis buffer, 50mM Tris-HCl (pH 8.0), 1% Tween-20 and 20mg/ml proteinase K
- Cycle tubes 95oC for 1 minute; 95oC for 15 seconds, 55oC for 15 seconds and 72oC for 10 seconds- 30 cycles; 72oC for 10 seconds to lyse cells and release DNA
- Samples can be stored at 4°C for short term or -20°C for longer term at this stage
- qPCR reactions are carried out in triplicate, with 3-5ul of template per reaction that is normally optimal, but may need to be optimized for the exact set up to be used
- Set up the qPCR reactions as described for mastermix to be used. These are generally 20 µl reactions utilizing 0.4µM primers and 0.2µM taqman probe.
- Ensure that all samples (e.g. those representing control cases and those representing patients) are distributed across the plate and that standard curves are kept the same to avoid plate bias
- Always run a negative control containing no sample DNA to ensure no contamination has occurred
- Run a standard curve for each plate
- Standard curve should consist of triplicates of serial 1/10 dilutions of sample DNA which can be total DNA from cultured cells or tissue samples, plasmids containing the region to be amplified, or larger PCR products covering the region for QPCR
- Seal each plate and spin briefly to avoid air bubbles from forming
- Set up qPCR cycles on a machine; the precise cycles will depend on the reaction to be used, but generally this is: 95°C for 60 secs, then 45 cycles of 95°C for 15 secs each, then 60°C for 30 secs
- Once cycles the are complete, analyse output for samples compared to standard curve
- Compare triplicates and outliers due to any pipetting error etc. can be removed

Additional things to consider:

- Ensure that sufficient standard curve material is available for the number of plates required as this will aid reproducibility of results, since all subsequent sample-based results will be compared to this
- It is best to optimize PCR reactions on standard curves first before working with precious samples
- Ensure that the qPCR Mastermix solution is suitable for the qPCR machine to be used (e.g. some require the inert fluorescent dye “ROX” to serve as a passive reference, while some do not)
- Ensure that the probe labels are of suitable fluorescence for the qPCR machine to be used and also that the correct quenchers have been selected
- If other material is not available for making a standard curve the regions of interest can be amplified from any human mtDNA containing cells, by using the following primers: MTND1 Fwd 5’ -CAGCCGCTATTAAAGGTTCG-3’ Rev 5’-AGAGTGCGTCATATGTTGTTC-3’, MTND4 Fwd 5’- ATCGCTCACACCTCATATCC -3’ Rev 5’-TAGGTCTGTTTGTCGTAGGC-3
- Products should be checked on an agarose gel and purified and concentration checked using standard methods
- Carry out all procedures at RT unless otherwise specified

Limitations of qPCR include measurement error when processing samples [Guyatt et al., 2019]. Starting DNA concentration is critical for copy number assays as low DNA concentrations can lead to increases in measurement error [Grady et al., 2014]. mtDNA heteroplasmy levels can also impact mtDNA CN assays with accuracy of the *MTND1/MTND4* assay decreasing when lower levels of deleted mtDNA molecules are present (Grady *et al.*, 2014). This makes studying mtCN challenging at a single cell level due to low starting concentrations of mtDNA and potentially high levels of heteroplasmy in individual cells. It is also difficult to measure subtle changes in mtCN or heteroplasmy levels with current qPCR technologies [Grady et al., 2014].

Performing qPCR in triplicate is standard but may not provide enough power to detect subtle changes in heteroplasmy levels, particularly when working with low starting mtDNA concentrations [Grady et al., 2014]. Using homogenate tissue samples to quantify mtDNA CN in

a specific cell type also carries the risk of measuring CN from other cell types, such as glia, that reside in the same tissues (Pyle et al., 2016).

There are a number of alternative methods to assess mtDNA copy number, many of these have downsides such as low throughput or high cost, but do offer an excellent way to confirm copy number and heteroplasmy differences on a smaller scale to compliment and confirm data that may have been obtained through QPCR. As mentioned above, mtDNA can be observed by immunofluorescence but this may be difficult to achieve when working with single cells derived from tissue samples. A more feasible method is southern blotting where total DNA is separated by electrophoresis and then blotted onto a membrane where selected regions can be identified by sequence specific radioactively-labelled probes [He et al., 2002]. This method offers a suitable method to confirm data achieved by QPCR, but at a single cell level material is very low and high accuracy is needed to observe differences. In addition this method is relatively low throughput, time consuming and requires the use of radioactive labels.

Other alternatives include microarray analysis and whole exome sequencing however, such methods are likely to be much more expensive and require specailist equipment.

An emerging alternative technique to qPCR is digital droplet PCR (ddPCR) which is more sensitive and can work with lower starting concentrations of DNA. Furthermore, ddPCR does not rely on the use of external standards and is thus considered more accurate than qPCR as there is a reduced occurrence of error rates. It has been shown that variation in copy number results can be seen with changes in DNA extraction kits and methods thus the key thing when setting up such experiments is to ensure and robust method is designed and tested before samples are tested and that all reagents and methods are kept the same for all samples. In addition a number of other qPCR assays are available to allow analysis of mutation

load such as the Random Mutation Capture (RMC) assay [Vermulst et al., 2008] and long-range PCR assays [Sanders et al., 2018]. These may be harder to achieve on small samples of DNA from single cells but it is worth assessing what may be achieved with the samples available so as much information can be gain from what may be very small and rare samples.

Conclusions and future directions

Over the last 25 years significant progress has been made into investigating the link between mitochondrial dysfunction and the progression of PD. This includes studies examining the impact of both inherited and somatic mutations within mtDNA. Given the potential and causal role of mtDNA variation in PD pathogenesis,
understanding reasons for preferential accumulation of mtDNA deletions and differential patterns of mtDNA CN variation across different cell populations as a result of PD appears crucial in light of recent work which highlighted cell-specific responses to PD.

Whilst work is ongoing in this area to characterise where deletions occur in the mitochondrial genome and the effects of these deletions on mitochondrial function, single cell studies are not without limitations. The low starting yields of mtDNA obtained from a single neuron combined with DNA degradation during storage can create difficulties in amplifying and sequencing mtDNA. DNA degradation is also likely to occur in post-mortem brain tissue that is non-fixed. Studies on post-mortem tissue in PD are further compounded where vulnerable neurons have already died and those remaining may exhibit some resistance to degeneration. Furthermore, any errors in sampling methodology or sequencing could lead to spurious associations between mtDNA variants and PD.

The use of replication cohorts still seems to be lacking in methodology descriptions in published reports, but attempts to recognise the effects of population stratification are increasing with recent studies not only considering the effect of gender on study results but also investigating other effects such as early or late onset of PD. As sample sizes increase, associations between PD and less common mtDNA variants are now being identified. Computational genomic tools such as MutPred are using entire mtDNA sequences to screen for rare mtDNA variants in PD, that are not present at high enough levels to be detected by haplogroup studies. Finally, studying somatic mutations as a single neuron level has provided insight into how mtDNA deletions differ between individual neurons and between different neuronal populations in PD. It should be noted that current studies using post-mortem brain tissue are only able to sample surviving neurons and not those that have already died, potentially skewing findings towards neurons with some protection against degeneration.

Abbreviations used:

α-synuclein, α-SYN; Acetylcholine, ACh; Adenosine triphosphate, ATP; Choline acetyltransferase, ChAT; Copy number, CN; Displacement, D; Deep-brain stimulation, DBS; Digital droplet PCR, ddPCR; Dopamine transporter, DAT; Electron transport chain, ETC; Haematoxylin & Eosin, H&E; Heavy, H; Horseradish peroxidase, HRP; 5-hydroxytryptamine, 5-HT; Laser capture microdissection, LCM; Lewy bodies, LBs; Lewy neurites, LNs; Light, L; Light- (or lagging-) strand, CR RNA, LC-RNA; Locus coeruleus, LC; Luxol Fast Blue, LFB; 1-methyl-4-phenylpyridinium, MPP+; Mitochondrial, mt; Non-coding regions, NCR; Norepinephrine, NE; Oxidative phosphorylation, OXPHOS; Pedunculopontine nucleus, PPN; Phosphate buffered saline, PBS; Polymerase γ, POLG; Random Mutation Capture, RMC; Region of interest, ROI; Reactive oxygen species, ROS; Ribosomal RNA, rRNA; Room temperature, RT; Single nucleotide polymorphisms, SNPs; Substantia Nigra pars compacta, SNpc; Subthalamic nucleus, STN; 3,3′,5,5′-Tetramethylbenzidine, TMB; Transcription factor A, mitochondrial, TFAM; Transfer RNA, tRNA; Tris-buffered saline, TBS; Vascular endothelial growth factor, VEGF; Wild-type, w

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Figures:

Figure 1:

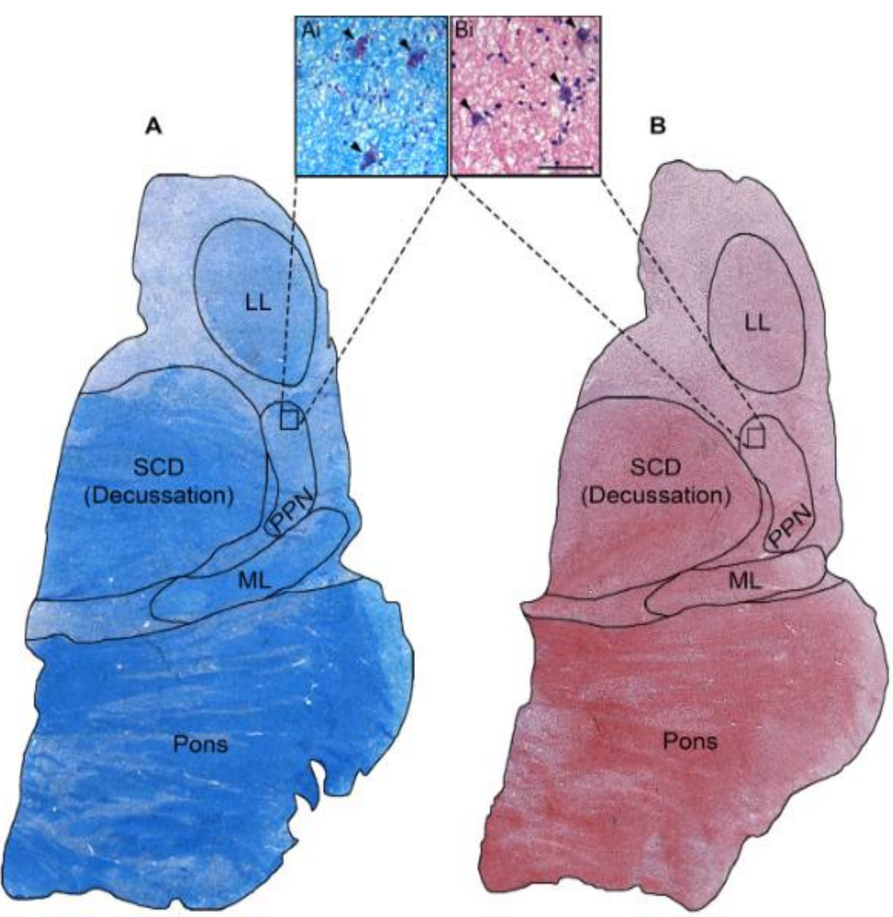

Figure 1. **(A)** LFB and **(B)** H&E-stained human post-mortem brain sections for confirming the presence of the ROI (in this case the PPN). Inset Ai & Bi depicts the cell type of intent (cholinergic neurons), showing their characteristically large and triangular shapes. The Figure was taken from a published report produced by our group [Bury et al. 2017].

Figure 2:

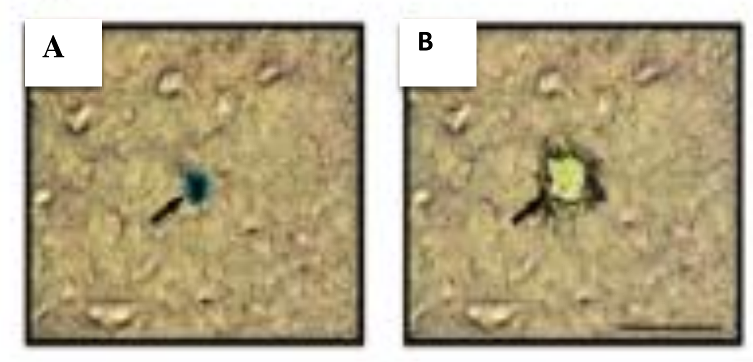

Figure 2. **(A)** A brain tissue section stained with a primary antibody to positively identify the neuronal type of interest, in this case targeting choline acetyltransferase (ChAT), a marker for cholinergic neurons, prior to cutting using an LCM apparatus. **(B)** A post-capture image after extracting the neuron from the surrounding tissue.

Figure 3:

A **B**

Figure 3. The UnipicL^{TM} apparatus, which offers a more economical cell capture technology than the more traditional laser-assisted target cell capture technologies. **(A)** The system is compatible with most inverted microscopes, to allow for rapid and efficient acquisition of specific cells from surrounding tissues or cell culture, with target cells that are collected based on their morphology, location or fluorescent label. **(B)** The major hardware components of the system are: (1) sampler head; (2) universal microscope straddle; (3) system alignment tool; (4) vacuum line and electrical cables; (5)control box with light intensity dial (top), vacuum strength dial (middle), vacuum impulse duration dial (bottom) and also the controls for the disposable capillary units.