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Ageing and Neurodegenerative Diseases

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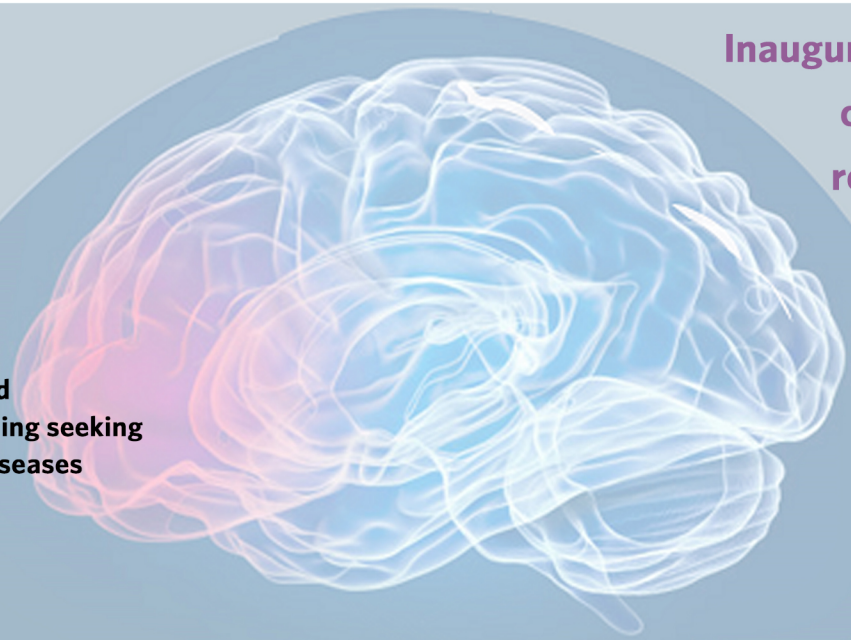

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Prof. Le is currently the deputy director of the Academic Committee and the director of the Institute of Neurology, Sichuan Academy of Medical Science-Sichuan Provincial People's Hospital. He is also a life-time Professor of Neurology and the director of Center for Translational Research on Neurological Diseases, the First Affiliated Hospital of Dalian Medical University. He has been engaged in clinical practice, teaching and research of neurology for almost 40 years, including 24 years of work in Baylor College of Medicine as a Professor of Neurology and Director of the Parkinson's Disease Research Center; and 10 years of working as a Professor of Neuroscience and Head of Neurogenomic Lab in the Institute of Health Science of Chinese Academy of Science jointed with Shanghai Jiao-Tong University School of Medicine.

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Journal Scope

High-quality original articles, reviews, case reports, commentaries are welcomed from ageing and neurodegenerative diseases, particularly on the basic mechanisms of ageing and their roles in the onset and progression of neurodegenerative diseases. The journal aims to report innovative research advances on the following topics:

1. The cellular and molecular mechanisms of ageing and the pathogenesis of neurodegenerative diseases;

2. The associations between neurodegenerative diseases and the biological bases of ageing with a focus on: genomic instability, epigenetic alterations, telomere attrition, protein degradation system failure, mitochondrial dysfunction, cellular senescence, nutrient sensing deregulation, stem cell exhaustion, intercellular communication impairment, etc.;

3. Translational research into prevention and treatment of age-related neurodegenerative diseases;

4. Mechanistic bases for epidemiological observations in aging-related neurodegenerative diseases.

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About the Journal

Ageing and Neurodegenerative Diseases (*AND*), ISSN 2769-5301 (Online) is an international peer-reviewed, open access, online journal. *AND* is a peer-reviewed and open access multidisciplinary journal that publishes high-quality original articles, reviews, case reports, commentaries, letters to editor, *etc*. Ageing is a major risk factor for neurodegeneration, and the prevalence of ageing-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, *etc*. continues to rise with the increased ageing population. Unfortunately, there are no effective treatments available for the age-related neurodegenerative diseases. Thus, to develop successful interventions, it is important to investigate the basic mechanisms of ageing and their roles in the onset and progression of neurodegenerative diseases.

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Ageing and
Neurodegenerative Diseases

Editorial

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Inauguration of a unique journal Ageing and Neurodegenerative Diseases: a new beginning seeking cures for age-related neurodegenerative diseases

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INTRODUCTION

As the Editor-in-Chief of the newly founded journal *Ageing and Neurodegenerative Diseases* (*AND*), I am honored to introduce this journal to you on behalf of the OAE Publishing Inc.

AND is a peer-reviewed and open access multidisciplinary journal publishing high-quality original articles, reviews, case reports, commentaries, letters to the editor, *etc.* The aims of this journal are to report innovative research advances in the cellular and molecular mechanisms underlying the ageing process and age-related neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, *etc*. We are also interested in publishing innovative research describing therapeutic interventions in this field. Through this journal, we aim to promote collaboration and interaction among basic scientists, clinicians, and industrial experts. Our ultimate goal is to find solutions for slowing the ageing process, which will hopefully ameliorate and/or delay the onset of neurodegenerative diseases.

AGEING POPULATION IS A BIG CHALLENGE TO OUR SOCIETY

According to the World Health Organization, nearly two billion people across the world are expected to be over 60 years old by 2050, which is twice the ageing population in 2000. With the increasing life expectancy,

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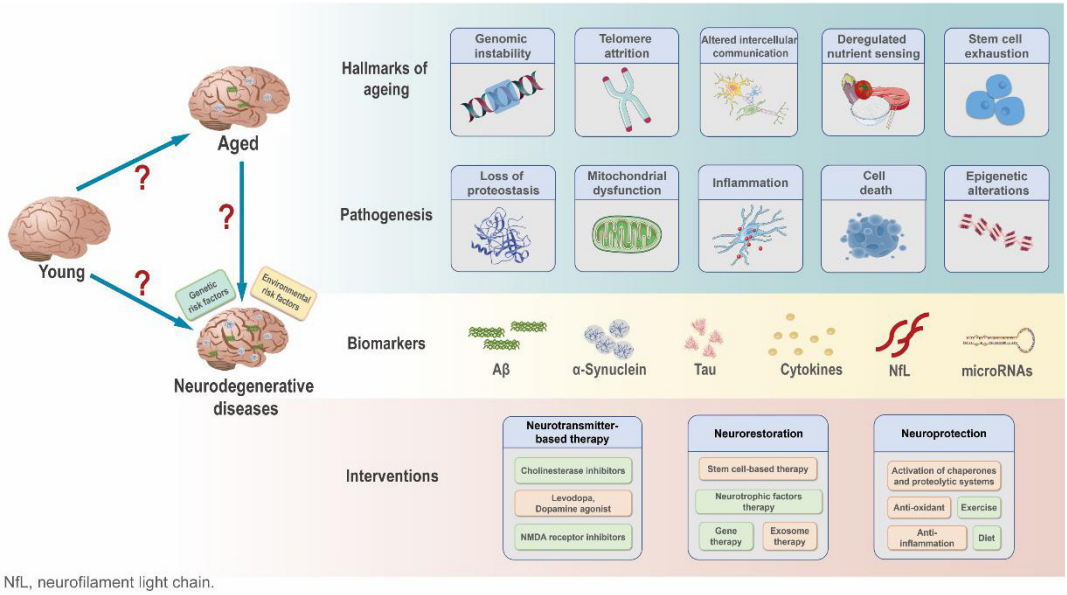

Figure 1. The hallmarks of ageing, the biomarkers and intervention for neurodegenerative diseases. The scheme enumerates the hallmarks of ageing, and displays the proposed pathogenesis, biomarkers and interventions for several neurodegenerative diseases.

the global ageing population has expanded rapidly in recent decades. In order to adapt to the increasing ageing population, many countries have raised the retirement age, reduced pension benefits, and have started spending more on elderly care. Health is the biggest issue that comes with ageing. Because of the increase in the ageing population, the prevalence of age-related chronic diseases has increased dramatically. These chronic diseases, including cardiovascular disease, diabetes, cancers, and neurodegenerative diseases, have become an urgent challenge, as there are currently no effective therapies for many of them. The governments in many countries have spent a significant resource to conduct research seeking solutions to delay the ageing process and reduce the incidence of most common age-related diseases.

UNCOVERING THE MECHANISMS UNDERLYING THE AGEING PROCESS IS AN URGENT TASK

Ageing is a natural process defined as the progressive deterioration of biological functions after the organism has attained its maximal reproductive competence. During the ageing process, our body suffers from a serious of metabolism abnormality and cell damage, which leads to the phenotypes of ageing, along with age-related diseases. Among the age-related diseases, neurodegenerative diseases have received a lot of attention due to their irreversibility, lack of effective treatments, and their associated social and economic burdens. Brain ageing has been considered to predispose to neurodegenerative disorders. At a cellular level, brain ageing is characterized by increased inflammation, oxidative stress, increased genomic instability, telomere attrition, epigenetic alterations, metabolism impairment, protein homeostasis disturbance, mitochondrial dysfunction, cellular senescence, nutrient sensing deregulation, stem cell exhaustion and intercellular communication blockage [Figure 1]. However, despite intensive research, the exact molecular mechanisms underlying the ageing process, particularly the molecular pathways and networks accounting for the switch from physiological brain ageing to neurodegeneration, remain to be fully elucidated. A better understanding of the genetic and non-genetic factors regulating the ageing process will greatly benefit the discovery of anti-ageing remedies and novel therapies for neurodegenerative diseases.

AGE-RELATED NEURODEGENERATIVE DISEASES ARE A LEADING CONCERN IN NEUROSCIENCE AND NEUROLOGY RESEARCH

The ageing society today is confronted with an epidemic of chronic diseases, among which neurodegenerative diseases present an ever-growing medical and social burden. Ageing is a major risk factor for neurodegeneration, and that the prevalence of age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, *etc.* has dramatically increased in recent decades. Unfortunately, no treatments have been shown to slow the neurodegeneration in patients with these diseases. Thus, to develop successful interventions, it is extremely important to investigate the basic mechanisms of ageing and their role in the onset and progression of neurodegenerative diseases, the results of which will facilitate the discovery of potential targets for novel therapies for neurodegenerative diseases. In addition, early clinical intervention is crucial for the management of patients with neurodegenerative diseases. However, the lack of specific biomarkers for their accurate diagnosis hinders early clinical diagnosis and intervention of these devastating diseases. Furthermore, the discovery and development of novel effective therapies for neurodegenerative diseases largely depends on reliable biomarkers of mechanism and target engagement to accelerate therapeutic development [Figure 1]. Thus, we plan to launch this new journal, which is aimed to stimulate and communicate the innovative research on age-related neurodegenerative diseases, and enhance the collaboration and interaction among basic scientists, clinicians and industrial experts. We welcome you to submit your papers to this unique and promising journal of *AND*.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and material

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None.

Conflicts of interest

The author declared that there are no conflicts of interest.

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Not applicable.

Consent for publication

Not applicable.

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Ageing and Neurodegenerative Diseases

Review

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Autophagy in ageing and ageing-related neurodegenerative diseases

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Abstract

Autophagy is a catabolic mechanism that allows cells to deliver cytoplasmic contents to lysosomes for degradation to maintain energy homeostasis and to protect cells against stress. Autophagy has been directly linked to neurodegeneration and ageing by an extensive body of research. It has become evident that disruption of autophagy contributes significantly to age-related pathologies and to the cognitive and motor declines associated with "healthy" ageing. Autophagic dysfunction causes the accumulation of many of the toxic, aggregate-prone proteins that are responsible for neurodegenerative diseases, including mutant huntingtin, alpha-synuclein, tau, and others. Since upregulation of autophagy has been found to reduce levels of such protein species, the therapeutic potential of autophagy induction as a strategy against age-related diseases and a method for modulating longevity has been widely studied. Here we review the evidence supporting a role for autophagy dysfunction in the progression of the age-associated functional decline in the brain and age-related brain pathologies and discuss the available evidence that upregulation of autophagy may be a valuable therapeutic strategy.

Keywords: Autophagy, ageing, neurodegenerative diseases, misfolded proteins, protein degradation

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INTRODUCTION

Autophagy is a term used to describe three pathways that deliver cytoplasmic contents to lysosomes for digestion and recycling. These include microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. The three pathways coexist in most mammalian cells and differ in their modes of delivery of cytoplasmic cargo to the lysosomal compartment. In brief, microautophagy involves direct delivery of cytosolic components to the lysosome by invagination of the lysosomal membrane[1], while CMA involves selective translocation of cytoplasmic proteins with a KFERQ peptide motif into the lysosomal lumen for degradation[2]. In macroautophagy, the cytoplasmic cargo, which can include proteins and organelles, is sequestered and engulfed by double-membraned, cup-shaped structures known as phagophores that elongate and extend around the intracellular content to form an autophagosome. Autophagosomes ultimately fuse with lysosomes to become autolysosomes, after which the degradative enzymes degrade the autophagic contents. In this review, we will focus on macroautophagy (hereafter referred to as autophagy).

Autophagy can be divided into selective and non-selective forms of autophagy[3]. Non-selective autophagy (often referred to as bulk autophagy) is considered in lacking cargo specificity and can be induced by various cellular stimuli, such as energy and nutrient deprivation. Selective autophagy increases the likelihood of cargo capture and can degrade aggregate-prone proteins (aggrephagy), intracellular pathogens (xenophagy), damaged mitochondria (mitophagy) and lysosomes (lysophagy), excess peroxisomes (pexophagy), and dysfunctional endoplasmic reticulum (ER-phagy).

Since it was first discovered in yeast as a survival mechanism[4], it has become evident that autophagy is important for a variety of biological processes. Interestingly, perturbation of both selective and non-selective forms of autophagy has been associated with ageing and numerous age-related disorders, such as inflammation, cancer, diabetes and neurodegenerative diseases[5].

The susceptibility of neurons to accumulate defective organelles and proteins due to their post-mitotic nature and the age-related decline in autophagic capacity appears to create an unfortunate paradox that puts autophagic dysfunction at the center of many neurodegenerative diseases where accumulation of toxic aggregated proteins is a hallmark and a causal factor for the disease pathogenesis'. Additionally, variants/polymorphisms in autophagy genes have been implicated in numerous age-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis/frontotemporal dementia (ALS/FTD) and may contribute to the accumulation of toxic intracellular protein aggregates in the brain. This has led to interesting questions regarding the potential of autophagy induction as a treatment against age-related diseases.

In this review, we will describe the evidence of autophagy dysfunction in ageing and age-related disorders and the potential of autophagy upregulation as a therapeutic strategy for the functional decline associated with ageing and neurodegenerative disorders.

AUTOPHAGY AND NEURODEGENERATIVE DISEASES

Intracellular protein misfolding and aggregation are common features of the abovementioned late-onset neurodegenerative diseases, also referred to as proteinopathies. The Mendelian mutations that cause proteinopathies frequently lead to toxic gain-of-functions in the disease-associated proteins, the expression of which correlate with disease severity. It has become increasingly evident that a given proteinopathy can be caused by various mutations that disturb different parts of the autophagic machinery [Figure 1 and Table 1]. Thus, it is critical to understand the cellular impact of the mutations that cause age-related neurodegenerative diseases. Additionally, since the toxic aggregated proteins associated with the

Table 1. Selected pathophysiological mechanisms related to autophagy impairment in AD, PD, HD and ALS

	Autophagosome formation	Cargo recruitment	AP-lysosome fusion	Lysosome function
AD	· PICALM depletion in AD brains inhibits autophagosome formation · Reduced Beclin-1 levels cause decreased autophagosome biogenesis · CCT complex impairment reduces autophagosome formation	· PICALM depletion inhibits cargo recognition	· Loss-of-function of PICALM impairs the fusion of autophagosomes with lysosomes in a VAMP8-dependent process · Reduced CCT/TRiC complex expression in AD brains blocks autophagosome delivery · Tau accumulation disturbs the integrity of lysosome membranes and impairs autophagosome maturation by inhibiting HDAC6	· Decreased CCT/TRiC complex level in AD brains blocks lysosomal degradation · APOE4 variant increases Rab5-mediated endocytosis and causes non-degraded lipids and proteins to accumulate in swollen lysosomes.
PD	· VPS35 mutation causes ATG9 mislocalisation and inhibits autophagosome formation · Overexpression of α-syn *in vitro* and *in vivo* results in compromised autophagosome biogenesis through Rab1 inhibition, causing mislocalisation of ATG9		· Inclusion bodies containing α-syn inhibit autophagosome maturation and fusion with lysosomes, resulting in decreased protein degradation	· Depletion of ATP13A2 leads to decreased SYT11, which results in lysosomal dysfunction · iPSC-derived neurons from PD patients with GBA mutations showed increased α-syn levels, as well as autophagic and lysosomal defects · Accumulation of α-syn aggregates in the nervous system causes lysosomal impairment · PD mutation in LRRK2 G2019S induces defective lysosomal positioning mediated by Rab7 and enlarged lysosomes containing nondegraded contents
HD	· mHTT impairs autophagosome biogenesis	· mHTT causes defects in cargo recognition by inhibiting organelle sequestration	· mHTT perturbs post-Golgi trafficking to lysosomes, leading to dysfunctional autophagosome/lysosome dynamics	
ALS		· ALS-associated L341V p62 exhibits defective binding to the core autophagy protein LC3 and impaired recruitment to autophagosomes · ALS/FTD-causing mutations of TBK1 impair binding and phosphorylation of optineurin, leading to reduced autophagy substrate recognition and clearance	· Truncated CHMP2B blocks autophagy flux in cultured primary neurons from rat cortex and photoreceptor neurons in *Drosophila* eye sections · R155H mutation of VCP disrupts clearance of damaged lysosomes by autophagy	
Ageing	· Reduced autophagosome formation in liver tissue from aged mice		· Aged *C. elegans* has a dysfunctional late autophagic flux	

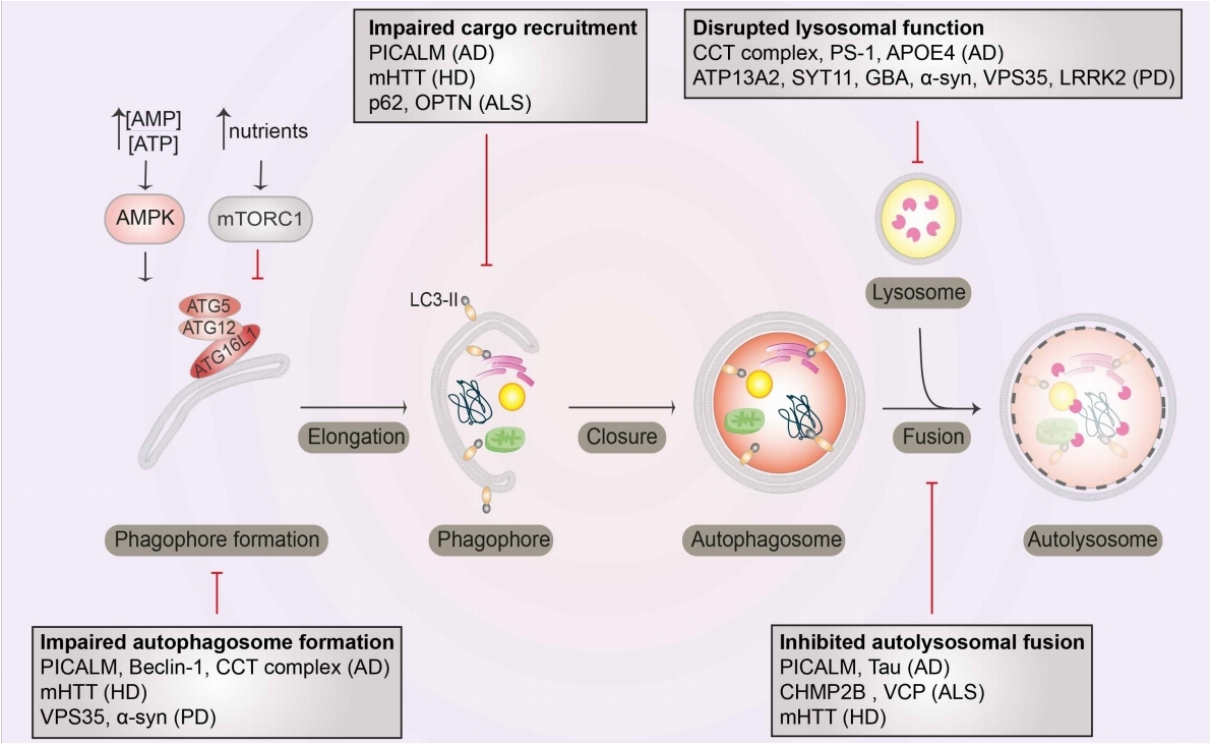

Figure 1. Disruption of autophagy machinery in neurodegenerative diseases. Numerous genes implicated in neurodegenerative diseases such as AD, HD, PD and ALS have been associated with autophagy. These genes disrupt different parts of the autophagic machinery, including autophagosome formation, cargo recruitment, lysosomal function or autolysosomal fusion.

proteinopathies are substrates for autophagy, upregulation of autophagy or prevention of the age-related functional decline in autophagy may prove to be valuable as a therapeutic strategy.

Alzheimer's disease

AD is a progressive neurodegenerative disorder characterized by two main pathological hallmarks: the extracellular deposit of amyloid-β (Aβ) plaques and the intracellular accumulation of hyperphosphorylated tau tangles[6]. There is a complex interplay between Aβ and autophagy, as autophagy controls both the clearance and generation of Aβ. On the one hand, upregulation of this clearance pathway can reduce the accumulation of amyloid plaques and rescue neurodegeneration in various systems[7-9]. On the other hand, Aβ may also be generated in autophagosomes, which appear to contain both the precursor [the amyloid precursor protein (APP)] and the enzyme Presenilin-1 responsible for the cleavage of APP to Aβ[7,10]. Furthermore, autophagy is critical for the extracellular secretion of Aβ peptides, as conditional knockout of the key autophagy gene, *Atg7*, in APP transgenic mice carrying the Swedish mutation was shown to cause less extracellular Aβ secretion and fewer extracellular Aβ plaques. However, these events were associated with toxic intracellular aggregation of Aβ, which likely causes neurodegeneration, and together with amyloidosis, memory deficit[11]. Besides Aβ, there is also a link between hyperphosphorylated tau and autophagy. Despite being substrates of the ubiquitin-proteasome system, tau proteins are delivered to the autophagosome-lysosomal system for degradation, as autophagy impairment leads to the formation of tau oligomers and accumulation of tau insoluble species[12]. Indeed, autophagy-inducing drugs like verapamil or felodipine induce the clearance of tau in a zebrafish system[13]. Furthermore, autophagy impacts tau phosphorylation: autophagy-deficient mice display hyperphosphorylated tau, while autophagy induction reduces tau phosphorylation[14-16]. Interestingly, tau *per se* modulates the autophagy pathway, with tau accumulation disturbing the integrity of lysosome membranes and retarding autophagosome maturation by

inhibiting the activity of histone deacetylase 6[15].

AD-associated mutations have been found to affect the three main steps of the autophagy pathways: autophagosome synthesis, trafficking, and maturation/degradation[17,18].

The expression of Beclin-1, an essential participant in autophagosome biogenesis, is decreased in the affected brain regions of AD patients compared to healthy individuals, likely due to its increased proteolytic cleavage mediated by caspase 3, which is highly activated in AD brains[19]. Since a decline in Beclin-1 activity leads to a decrease in autophagosome synthesis, this is associated with ageing and neurodegeneration[20].

Genome-wide association studies in AD identified variants of phosphatidylinositol binding clathrin assembly protein (PICALM), which also shows accelerated cleavage and, consequently, loss-of-function, in AD[21,22]. PICALM is a clathrin adaptor protein, whose loss-of-function impairs endocytosis of VAMP2 and VAMP3 (soluble NSF attachment protein receptors - SNAREs involved in the fusion of autophagosome precursors), as well as VAMP8 (involved in the fusion of autophagosomes with lysosomes/late endosomes). Thus, PICALM depletion in AD brains inhibits autophagy at multiple steps: from autophagosome biogenesis to maturation and fusion steps, ultimately leading to tau accumulation[17,23].

Studies based on mouse and cell models of AD have reported that the trafficking of autophagy-related compartments is selectively defective[24]. Inhibiting the microtubule-mediated retrograde transportation of autophagosomes towards lysosomes (from the distal axonal regions towards the soma) leads to the rapid accumulation of immature autophagosomes in neurites, a morphology similar to that observed in the AD brains[7]. Tau is known to be critical for this retrograde vesicle trafficking as it regulates the assembly and stabilization of neuronal microtubules[25]. Importantly, tau gains a toxic function in AD models, generating neurofibrillary tangles, which may negatively impact neuronal vesicle trafficking[26]. Reduced expression of the chaperonin CCT/TRiC complex, an important chaperone that assists the proper folding of tau, was found in the affected brain regions of AD patients[27,28]. Apart from its direct action on tau, CCT is essential for maintaining the cytoskeleton structure in neurons and consequently to ensure proper trafficking of autophagosomes and their ultimate fusion with lysosomes. CCT impairment, by blocking autophagosome delivery/degradation, causes the accumulation of long-lived proteins and autophagy substrates, such as p62[27].

In addition to the aforementioned factors regulating autophagic flux (PICALM and CCT), mutations of Presenilin-1 (PS1), known to cause early-onset familial Alzheimer's disease, impair autophagosome maturation/degradation via dysfunction of the lysosomal V-ATPase in patient-derived cells[29]. Besides its role in the APP cleavage to Aβ as part of the γ-secretase complex, PS1 regulates N-glycosylation of the V-ATPase subunit V_oA1 essential for lysosomal acidification and subsequent degradation of autophagic content. Abnormally increased levels of acid sphingomyelinase (SMase) in AD brains lower TFEB levels by promoting its proteolysis[30]. Conversely, chemical inhibition of this SMase with amitriptyline increased the degradation of p62 and LC3-II, and delayed the disease development in APP/PS1 mouse models of AD[17,30]. Additional genetic risk factors for AD, such as the *APOE4* variant, mutant APP or APP duplication, increase Rab5-mediated endocytosis and cause non-degraded lipids and proteins to accumulate in swollen lysosomes. Indeed, AD mice models show lysosomal dysfunction with subsequent accumulation of autophagy substrates[7,10].

Autophagy modulation as therapy in AD

Autophagy induction may be a promising therapeutic strategy in AD [Table 2]. Autophagy stimulation can be achieved either chemically [using mechanistic target of rapamycin (mTOR)-dependent or -independent small molecule autophagy enhancers] or through gene therapy approaches, as summarised below[6,31].

The first group of chemical compounds, which inhibit the negative autophagy regulator mTOR, such as rapamycin and other rapalogs (eg. temsirolimus), reduce the levels of hyperhosphorylated tau and Aβ, and consequently ameliorate the cognitive and memory deficits in multiple AD model mice (such as P301S mutant tau and PDAPP transgenic mouse models[32,33]), likely due (at least in part) to autophagy activation in neurons[34]. Rapamycin also reduces tau toxicity and increases the life-span of *Drosophila (Drosophila Melanogaster)* expressing wildtype or mutant R406W tau, as a result of enhanced autophagic clearance of soluble and insoluble tau[35].

The second group of compounds mainly comprises the class of small molecule enhancers of autophagy that often signal through AMPK, which directly phosphorylates the AMPK substrate ULK1 and thereby induces autophagy[36]. These molecules may be either direct AMPK modulators (such as metformin, bosutinib, nilotinib) or indirect upstream activators acting by lowering mitochondrial ATP production and/or intracytoplasmic Ca^{2+} levels[37,38]. The direct AMPK modulators show beneficial effects in multiple transgenic APP mouse models[39,40] and prevent the degeneration of neurons producing dopamine, a neurotransmitter involved in the regulation of cognitive and non-cognitive functions in a *Tg2576* AD mouse model[41]. Thus, several of these AMPK-stimulating compounds have progressed to pilot clinical studies for AD, where metformin has shown promising early results with improved memory and executive function[42,43].

The second group of compounds comprises a continuously growing list of molecules. Among them, the disaccharide trehalose activates AMPK by lowering ATP production by inhibiting GLUT transporters, and was shown to enhance the clearance of tau aggregates[44-46]. The clearance of autophagy substrates can also be upregulated by a class of mood-stabilizing drugs, including lithium[45,47]. Lithium administration results in lowering of the Ca^{2+} levels in the cytoplasm by reducing the intracellular levels of IP_3, decreasing the mitochondrial uptake of Ca^{2+} and consequently ATP production, causing AMPK activation[48]. Lithium additionally upregulates autophagy by inhibiting GSK3β, reducing the phosphorylation of tau and the formation of Aβ plaques in pre-pathological AD mouse models (APPswe/PS1A246E)[49]. In clinical trials, lithium treatment showed reduced cognitive decline over a two year period compared to the placebo group, accompanied by enhanced Aβ clearance from the brain[50]. Other molecules that likely signal through the IP_3-Ca^{2+}-AMPK axis include the class of imidazoline receptor agonists, such as clonidine and rilmenidine[51]. Treatment with these two drugs increase the clearance of mutant tau and ameliorate the motor and morphological defects in an experimental zebrafish model expressing the human A152T disease-associated tau variant[52]. Another class of compounds, the L-type Ca^{2+} channel antagonists (felodipine and verapamil) block the influx of extracellular Ca^{2+} and likely activate autophagy via the aforementioned Ca^{2+}-AMPK axis. Consequently, apart from their antihypertensive effects, these drugs improve the clearance of insoluble tau and ameliorate the morphological defects seen in two transgenic zebrafish tauopathy models[53]. Elevated intracytosolic Ca^{2+} levels activate calpains, which inhibit autophagy. The calpain inhibitor A-705253 improves clearance of hyperphosphorylated tau and Aβ and has beneficial cognitive effects in a triple transgenic AD mouse model (bearing disease-associated mutations in three genes: PS1M146V, APPswe, TauP301L)[54].

While the described chemical compounds that upregulate autophagy offer clear beneficial effects in multiple AD *in vivo* models, it is important to acknowledge that many of these compounds may also exert beneficial

Table 2. Therapeutic strategies to modulate autophagy and their effects on disease models (see main text for references)

Mechanisms of drugs	Examples
Rapamycin: mTOR inhibition	AD: Rapamycin and other rapalogs reduce the levels of hyperhosphorylated tau and Aβ, and improve cognitive and memory deficits in AD mice and *Drosophila*
	PD: Rapamycin treatment increases the clearance of mutant α-syn and attenuates toxicity in PC12 cells, dopaminergic neurons and PD animal models
	HD: Rapamycin and its derivatives facilitate clearance of PolyQ aggregates and reduce toxicity in HD *Drosophila* and mice
	ALS: Rapamycin treatment decreases TDP-43 inclusions and forebrain neurodegeneration in a *FTD* mouse model. Rapamycin also reduces neuronal TBPH aggregates in an ALS *Drosophila* model, as well as partially rescuing lifespan and locomotor defects in these animals
	Ageing: Rapamycin increases mortality in mice and life extension in *Drosophila*
SMERs: mTOR-independent autophagy	PD: SMERs induce autophagic clearance of A53T α-syn *in vitro* independently of mTOR
	HD: SMERs trigger the elimination of mHTT aggregates and ameliorate toxicity in cell culture and in HD *Drosophila* models
Metformin, bosutinib, nilotinib: AMPK activation	AD: Metformin, bosutinib and nilotinib prevent the degeneration of dopaminergic neurons, the loss of which affects cognitive and non-cognitive functions in a Tg2576 AD mice
	PD: Metformin reduces levels of phosphorylated α-syn Ser129 and rescues mitochondrial dysfunction in SH-SY5Y and *Drosophila* models Nilotinib partially rescues disease phenotypes in both A53T and PD mice and improves cognitive and motor skills in PD patients Resveratrol induces α-syn clearance, possibly via interaction with SIRT1 in SH-SY5Y and PC12 cells
	HD: Metformin significantly clears PolyQ aggregates and improves motor and behavioural dysfunction in PolyQ disease mice
Trehalose: AMPK activation	AD: Trehalose induces tau clearance and neuronal survival in AD *tauP301S* mice
	ALS: Trehalose ameliorates early motor phenotypes in the G93A SOD-1 ALS mice, but fails to delay end-stage motor phenotypes
	HD: Trehalose enhances the degradation of mHTT aggregates in HD mice
Lithium, Clonidine, rilmenidine: IP_3-Ca^{2+}-AMPK axis	AD: Lithium reduces tau phosphorylation and the formation of Aβ plaques in pre-pathological AD mice. Clonidine and rilmenidine induce mutant tau clearance and ameliorate motor defect in A152T tau zebrafish
	PD: Lithium increases the clearance of A53T and A30P mutant α-syn in inducible PC12 cell models and ameliorates phenotypes associated with PD in aged Parkin mutant tg mice
	HD: Clonidine enhances removal of aberrant mHTT aggregates in HD in an mTOR-independent manner
Felodipine, verapamil: Ca^{2+}-AMPK axis	AD: Felodipine and verapamil improve the clearance of insoluble tau and ameliorate the morphological defects seen in two transgenic zebrafish tauopathy models
	PD: Felodipine decreases the levels of A53T α-syn in a PD mouse model and ameliorates neurodegeneration
	HD: Felodipine decreases mHTT in a HD mouse model and ameliorates signs of disease
A-705253: Calpain inhibition	AD: A-705253 improves clearance of hyperphosphorylated tau and Aβ and has beneficial cognitive effects in 3xtg AD mice
	HD: Downregulation of Calpain decreases mHTT aggregates and is neuroprotective in HD mice and *Drosophila*

effects via autophagy-independent mechanisms. Thus, targeting key autophagy genes through genetic approaches may enable more specific therapeutic strategies.

One of the most common strategies to genetically stimulate autophagy is by lentiviral-mediated overexpression of *BECN1*, which encodes Beclin-1[19]. This strategy ameliorates the amyloid pathology in the hippocampus and cortex of APP transgenic mice. Beclin-1-mediated autophagy has also been successfully induced by lentiviral Parkin transduction and was shown to clear intracellular Aβ in a triple transgenic AD mouse model (mutations in three genes: *PS1M146V*, *APPswe*, *TauP301L*)[55]. Another elegant approach, using a knock-in point mutation in *BECN1*(F121A) that blocks the interaction with its canonical inhibitor Bcl-2, causes a significant reduction in Aβ levels and increases the life-span of *5xFAD* mice (expressing a combination of 5 familial AD mutations in human *PS1* and *APP* genes)[56]. Similar to *BECN1* overexpression, ATG5-induced autophagy ameliorates the morphological defects and enhances the tau clearance in an A152T tau zebrafish model[52].

AD is a complex disease that results in impairment at multiple steps in the autophagy pathway, from autophagosome biogenesis to lysosomal functioning. In the AD scenarios with compromised autophagosome degradation within lysosomes, overexpression of *BECN1* and *ATG5* genes mainly induces the formation of autophagosomes, with little effect on lysosomal biogenesis, and has the theoretical potential to lead to the accumulation of nondegraded autophagosomes, which may further worsen pathology (depending on the extent of defective autophagosome clearance). To improve lysosomal function, studies using genetic ablation of cystatin B (an endogenous inhibitor of lysosomal cysteine proteases) successfully alleviated the memory deficits and amyloid pathology in a *TgCRND8* mouse model of aggressive AD amyloidosis[57]. Thus, combined approaches that act to enhance overall autophagy flux, by simultaneously upregulating the autophagosome synthesis and improving the lysosomal functioning, would be ideal therapeutic strategies. Therefore, an interesting target for drug development may be TFEB, as this master transcription factor controls the synthesis of both autophagosomes and lysosomes[38,58]. Indeed, TFEB overexpression improves the behavioural defects and enhances the clearance of hyperphosphorylated tau in a *rTg4510* mouse model of tauopathy[59].

Parkinson's disease

PD is the most common neurodegenerative movement disorder, primarily associated with progressive loss of motor control, often accompanied by cognitive decline. It is characterized by the presence of intraneuronal inclusions known as Lewy bodies and Lewy neurites enriched with filamentous forms of α-synuclein (α-syn)[60], which is encoded by *SNCA*. Multiplications of the *SNCA* locus cause autosomal dominant forms of PD. Levels of α-syn correlate with disease severity[61]. Defective autophagy has been implicated as central in the aetiology and pathogenesis of PD. Similar to AD, mutations causing PD appear to affect different stages of the autophagy itinerary, such as autophagosome biogenesis or fusion with lysosomes[62].

The presence of inclusion bodies containing α-syn affects autophagosome maturation and fusion with lysosomes, resulting in decreased protein degradation[63], suggesting that the presence of α-syn compromises autophagic flux. Overexpression of α-syn *in vitro* and *in vivo* results in compromised autophagosome biogenesis through the inhibition of Rab1, causing mislocalisation of ATG9[64]. A mutation in *VPS35*, which encodes a component of the retromer complex, results in an autosomal dominant form of PD, impairs autophagy, causes ATG9 mislocalisation and inhibits autophagosome formation[65]. A recent study in an α-synucleinopathy *Drosophila* model suggests that α-syn expression impairs autophagic flux in ageing adult neurons by disrupting the F-actin cytoskeleton[66].

There is increasing evidence of lysosomal defects in PD. Lysosomal impairment has been shown to cause accumulation of α-syn aggregates in the nervous system[67]. Autosomal dominant mutations in the *LRRK2* gene appear to affect endosome-to-lysosome trafficking. Overexpression of mutant lrrk, analogous to the most common PD causing mutation in human LRRK2 (G2019S), led to defective lysosomal positioning mediated by Rab7 and enlarged lysosomes containing nondegraded contents[68].

Heterozygous mutations in the gene encoding the lysosomal enzyme glucocerebrosidase (GBA) are the most commonly known genetic risk factors for PD. Induced pluripotent stem cells-derived neurons from PD patients with *GBA* mutations showed increased α-syn levels, as well as autophagic and lysosomal defects[69].

Furthermore, the PD-associated genes *ATP13A2* and *SYT11* regulate autophagy through a pathway mediated by TFEB[70]. Depletion of ATP13A2 *in vitro* has been shown to decrease levels of SYT11. The

decrease in SYT11 can account for the lysosomal dysfunction and impaired autophagosome degradation resulting from ATP13A2 deficiency, since SYT11 overexpression in ATP13A2 knockdown cells was able to rescue the autophagy defects in these cells[70].

Autosomal recessive mutations in *PINK1* or *Parkin*, two genes encoding proteins that are key regulators of a form of mitophagy, cause early onset Parkinsonism[71]. While these genes have led to suggestions that impaired mitochondrial quality control may contribute to PD, we have not focussed on this domain in this review focussing on ageing neurodegenerative diseases, as these Mendelian mutations manifest with disease in younger patients.

Autophagy modulation as therapy in PD

Inhibition of mTOR with rapamycin treatment increases the clearance of WT and PD-related mutant forms (A30P and A53T) of α-syn in PC12 cells[72], thereby attenuating toxicity in both dopaminergic neurons[73] and animal models of PD [Table 2][74,75].

Various mTOR-independent autophagy inducers have shown beneficial effects in PD models. Several novel small-molecule enhancers of rapamycin (SMERs) - SMER10, SMER18, and SMER28 - were found to induce autophagic clearance of A53T α-syn *in vitro* independently of mTOR[76].

Another group of compounds that have shown beneficial effects in PD are AMPK activators. Metformin, a drug currently used in the treatment of type 2 diabetes, reduces levels of Ser-129 phosphorylated α-syn in SH-SY5Y cells expressing α-syn[77] and partially rescues the mitochondrial dysfunction in genetic *Drosophila* models of PD[78]. Nilotinib, a tyrosine kinase inhibitor, induces autophagy, and partially rescues disease phenotypes in both A53T and lentiviral α-syn overexpression PD mouse models by inhibiting phosphorylation of BCR-ABL[79]. Clinical trials of nilotinib in PD patients showed improved cognitive and motor skills[80]. Resveratrol, a natural plant phenol, induces AMPK-dependent autophagy and α-syn clearance in SH-SY5Y and PC12 cells, possibly via SIRT1, a histone/protein deacetylase that activates autophagy via deacetylation of autophagy proteins[81-83]. The mTOR-independent autophagy inducer lithium increases the clearance of A53T and A30P mutant α-syn in inducible PC12 cell models[47] and reduces phenotypes associated with PD in an aged Parkin mutant transgenic mouse model[84]. In addition to lithium, other mood-stabilizing drugs, such as sodium valproate and carbamazepine, have been shown to induce autophagy in SH-SY5Y cells exposed to rotenone-induced toxicity[85]. Upon treatment with trehalose, autophagy was induced and enhanced clearance of α-syn both *in vitro*[86] and *in vivo*[87]. Additionally, felodipine decreases the levels of A53T α-syn in a mouse model of PD and ameliorates neurodegeneration[13].

Huntington's disease

HD is a heritable progressive neurodegenerative disease categorized as a polyglutamine (PolyQ) disease, along with other diseases such, dentatorubralpallidoluysian atrophy, spinal and bulbal muscular atrophy (SBMA), spinocerebellar ataxias (SCA) types 1, 2, 6, 7, 17, as well as Machado-Joseph disease (MJD/SCA3)[88]. Most PolyQ diseases are autosomal dominant except for SBMA, which is an X-linked disorder characterised by neurological, psychiatric and motor symptoms[89].

Each of these PolyQ diseases are caused by abnormal CAG repeat expansions that encode a PolyQ tract within specific genes. The lengths of PolyQ tracts vary in the different PolyQ diseases and cause formation of toxic oligomers and aggregates. Moreover, the length of the PolyQ tracts negatively correlates with the age of disease onset[90]. PolyQ diseases have been associated with dysfunctional autophagy and an altered

endo-lysosomal network required for the elimination of aggregates[91].

HD, the most common PolyQ disease, results from an abnormal CAG repeat expansion (> 35 repeats) encoding a PolyQ tract at the N-terminus of the huntingtin (*Htt*) gene[92]. PolyQ expansions in mutant huntingtin (mHTT) promote the accumulation of perinuclear cytoplasmic aggregates and intranuclear inclusion bodies in neurons[93,94,95]. Defects have been observed at different stages of the autophagy itinerary in Huntington's disease. The non-aggregated mutant huntingtin impacts starvation-induced autophagosome biogenesis by impairing the ability of wildtype ataxin-3 to deubiquitinate Beclin-1 and protect this protein from proteasomal degradation[96]. HD is also associated with defective autophagic cargo recognition[97]. Wildtype HTT acts as a scaffold protein which interacts with autophagy components to facilitate the degradation of selective autophagy cargos[98,99]. Defects in cargo recognition caused by mHTT inhibit organelle sequestration through autophagy and consequently result in abnormal intracellular lipid stores and altered mitochondria in mHTT mice[97]. HTT plays an important role in the regulation for axonal transport of autophagosomes via huntingtin-associated protein-1 (HAP1). In particular, loss of HTT or HAP1 disrupts the retrograde axonal transport of autophagosomes[100]. Retrograde transport is controlled by HTT and the microtubule motor protein dynein. Suppression of dynein inhibits autophagosome-lysosome fusion and aggravates the phenotype in HD *Drosophila* and mice[101]. Moreover, phosphorylation of HTT by AKT controls anterograde transport via kinesin1 recruitment[102]. Indeed, impaired autophagosome dynamics by mHTT expression also results in the inefficient degradation of dysfunctional mitochondria and mHTT aggregates in HD neurons, indicating that HTT controls its own elimination[100,101]. In addition, mHTT is involved in the regulation of post-Golgi trafficking. mHTT reduces the interaction with the optineurin/Rab8 complex and subsequently perturbs post-Golgi trafficking to lysosomes, which could result in dysfunctional autophagosome/lysosome dynamics[103]. A striatal-specific protein, Rhes, directly binds mHtt and accelerates mHTT-mediated cytotoxicity[104]. In the striatum, Rhes binds to Beclin-1 and promotes the dissociation of Bcl-2 and Beclin-1, leading to autophagy activation. However, mHTT expression interferes with Rhes-mediated autophagy induction[104,105]. Lower levels of Rhes are observed in HD, whereas overexpression of Rhes ameliorates HD-associated phenotypes in mice[105]. The V271A polymorphism in the core autophagy gene *ATG7* is associated with earlier onset of HD, suggesting that ATG7 may be implicated in HD, although the exact mechanisms associated with this polymorphisms have yet to be fully elucidated[106]. In addition, PolyQ tracts in mHTT impair mitophagy and have been associated with increased oxidative stress and the accumulation of damaged mitochondria[107]. Interestingly, compensatory upregulation of CMA is observed in the early stages of HD, possibly as response to macroautophagic dysfunction[108].

Autophagy modulation as therapy in HD

Pharmacological and genetic approaches to upregulate autophagy in PolyQ diseases have shown that increased clearance of PolyQ aggregates via autophagy can ameliorate PolyQ disease pathology [Table 2][91]. In contrast, inhibition of autophagy has been shown to cause cytotoxicity as well as increased polyQ aggregates accumulation *in vivo*[109].

Depletion of mHTT itself in symptomatic HD mice significantly reduced aggregation in the brain. Deletion of the PolyQ tract within HTT (ΔQ-htt) induces mTOR-independent autophagy in neurons. Interestingly, expression of ΔQ-htt in PolyQ-HTT knock-in (Hdh140Q/+) mice has protective effects, decreasing HTT neuropil aggregates, mitigating motor and behavioural defects as well as promoting longevity[110]. mTOR-dependent autophagy activation has been shown to have beneficial effects in PolyQ disease. Rapamycin and its derivatives reduced toxicity. In addition, the rapamycin analogue temsirolimus facilitated clearance of PolyQ aggregates mediated by autophagy activation and rescued pathology in HD *Drosophila* and mice[109,111]. mTOR-independent autophagy activation via trehalose enhances the degradation of mHTT

aggregates. Combination treatment of rapamycin and trehalose synergistically accelerated clearance of mHTT aggregates in HD *Drosophila*[86]. SMER10, 18, and SMER28, mTOR-independent autophagy inducers, trigger the elimination of mHTT aggregates and ameliorate toxicity in cell culture and in *Drosophila* HD models[76]. Interestingly, dual treatment SMERs with rapamycin had enhanced protective effects. Lithium treatment decreased soluble and aggregated mHTT, correlating with decreased toxicity[112]. Induction of autophagy in both an mTOR-independent and -dependent manner using a combination therapy of lithium and rapamycin leads to protection against neurodegeneration in an HD *Drosophila* model[112]. The G_i signalling activator clonidine, which induces mTOR-independent autophagy and regulates cAMP or IP_3, enhances removal of aberrant mHTT aggregates in HD[51]. Other autophagy inducers such as berberine, rilmenidine, and metformin significantly clear PolyQ aggregates and improved motor and behavioural dysfunction in PolyQ disease mice[113-115].

Downregulation of calpain induces autophagy, decreasing the number of mHTT aggregates and consequently has neuroprotective effects in HD mice and *Drosophila*. Although calpain inhibition is neuroprotective in HD mouse models, prolonged brain overexpression of calpastatin, a calpain inhibitor, does not cause obvious deleterious effects in mice[116]. Depletion of the autophagy adaptor protein Alfy exacerbates mutant huntingtin toxicity in mice and HD patient-derived neuronal model[117]. Conversely, overexpression of Alfy induces selective degradation of aggregated proteins as well as neuroprotection in neuronal and *Drosophila* HD models, suggesting that Alfy-mediated selective autophagy alleviates HD neuropathology[117,118]. Thus, improved cargo recognition by autophagy adaptors may be a potential target for therapeutic strategy.

Expression of TFEB, which promotes lysosome biogenesis and autophagy, leads to increased lysosomal activity in the striatum of HD mice, resulting in decreased levels of mHTT[119]. In addition, a previous study reported that both macroautophagy and CMA function in the degradation of the Htt fragment containing amino acids 1-552. Interactions between mHTT and the CMA components heat shock protein cognate 70 and lysosome-associated protein 2A (LAMP-2A) enable the uptake and lysosomal degradation of Htt-552, whereas inhibition of the CMA pathway suppresses Htt-552 degradation[120]. Acetylation of mHTT by CBP activation or HDAC1 inhibition triggered mHtt degradation and enhanced neuroprotection through the autophagic-lysosomal pathway in primary neurons and *Caenorhabditis elegans (C. elegans)* HD model[121,122].

ALS/FTD

In recent years, the neurodegenerative diseases ALS and FTD have become widely accepted as divergent presentations of a common neurodegenerative disease process[123]. ALS is characterised by loss of lower motor neurons from the brainstem/spinal cord and upper motor neurons from the motor cortex, while FTD involves loss of neurons from the prefrontal and temporal cortices. However, many patients with ALS exhibit symptoms traditionally associated with FTD (cognitive impairment and behavioural changes) and patients with FTD can develop motor neuron involvement resulting in progressive muscle weakness[123].

ALS/FTD are age-related diseases, typically manifesting in the fifth or sixth decade of life[124]. Conversely, ALS transgenic mouse models are reported to exhibit abnormalities in axonal transport, sensorimotor development and neuronal excitability as early as the neonatal period, despite not exhibiting neurodegenerative phenotypes until mature adulthood[125-127]. This observation has prompted speculation that normal ageing is a prerequisite for the ALS/FTD neurodegenerative disease process[124].

One possible explanation is that normal age-related phenotypes exhibited by relevant cell types increase vulnerability to ALS/FTD pathology[128]. Relevant to ALS, various motor neuron deficits have been

associated with normal ageing. Motor neuron number decreases in older rats (22 months old compared with 6 months old)[129] and humans (diminished motor neuron population evident over 60 years old)[130]. Energy homeostasis is also impaired, with motor neurons from aged mice demonstrating less efficient electrophysiological properties (20 months old compared with 12 months old)[131] and motor neurons from elderly human donors (68-99 years old) exhibiting mitochondrial dysfunction[132]. These age-related phenotypes and others are likely to lower the susceptibility threshold for ALS/FTD-specific neurodegeneration.

Reduced autophagy in skeletal muscle is another factor liable to increase vulnerability to ALS pathology as part of normal ageing. Central to less "neuron centric" models of ALS pathology is the notion that skeletal muscle is structurally and functionally interconnected with lower motor neurons. Decreased lysosomal activity leading to impaired autophagic flux and lower expression of core autophagy proteins (ATG7 and LC3) is reported in skeletal muscle from older mice[133,134], with ATG7 and LC3 expression also decreased in skeletal muscle from sedentary elderly human donors[134].

Around 5%-10% of ALS and 30%-50% of FTD cases are familial, with mutations in more than 25 genes associated with ALS/FTD[135]. Sporadic and familial ALS/FTD are clinically identical. Models based on disease-causing mutations have implicated dysfunction at several points in the autophagy pathway in ALS/FTD pathogenesis. For instance, ALS-linked mutations in the autophagy receptor optineurin (Q398X and E478G) are reported to block autophagosome-lysosome fusion by disrupting myosin VI-mediated lysosomal trafficking in motor neuron-like NSC-34 cells[136]. Phosphorylation by the protein kinase TBK1 has been shown to improve the efficiency of optineurin as an autophagy receptor, thereby increasing autophagy substrate clearance[137]. ALS/FTD-causing mutations identified in TBK1 that impair binding and phosphorylation of optineurin[138,139] are therefore expected to reduce autophagy substrate recognition and clearance. Numerous ALS/FTD-linked mutations have also been identified in the autophagy receptor p62 (also known as SQSTM1)[140-142], with ALS-associated L341V p62 exhibiting defective binding to the core autophagy protein LC3 and impaired recruitment to autophagic vesicles in NSC-34 cells[143].

Other ALS/FTD-causing mutations linked to autophagy include a splice-site mutation in the ESCRT (endosomal sorting complex required for transport) protein CHMP2B, which produces a truncated final protein[144,145]. Truncated CHMP2B blocks flux through the autophagy pathway in cultured primary neurons from rat cortex and photoreceptor neurons in fly eye sections, resulting in autophagosome accumulation[145]. The ATPase p97 (also known as VCP) also exhibits an ALS/FTD-linked mutation (R155H) that disrupts clearance of damaged lysosomes by autophagy. MEFs and muscle fibres from human donors expressing R155H p97 consequently accumulate defective lysosomes[146]. R155H p97 and A232E p97 (another disease-related mutation) additionally drive accumulation of stress granules (nontranslating messenger ribonucleoprotein aggregates) in HeLa cells, suspected to result from defective autophagy[147]. Very recently, p97 has been shown to function in early autophagy initiation through the core autophagy protein Beclin-1[148]. Models based on disease-causing mutations therefore indicate defects in autophagy substrate recognition, autophagosome biogenesis and autophagosome-lysosome fusion, depending on the functional effects of the mutations on VCP activities.

Cytosolic protein aggregates, which are autophagy substrates, are found almost universally in ALS/FTD and typically comprise TDP-43 (TAR DNA binding protein 43), SOD-1 (superoxide dismutase 1), FUS (fused in sarcoma) and DPR (dipeptide repeat) proteins in various combinations[149]. Tau, p62 and ubiquitin can also feature in these cytosolic inclusions. Consequently, further experimental evidence linking autophagy to ALS/FTD stems from autophagic degradation of ALS/FTD-associated aggregate-prone proteins.

A hexanucleotide (GGGGCC) repeat expansion in an untranslated region of the C9ORF72 transcript is the most common genetic abnormality identified in familial and sporadic ALS/FTD[150-152]. Hexanucleotide repeat expanded C9ORF72 generates DPR proteins, which adopt aggregate-prone conformations and form cytosolic inclusions[153]. Work in motor neuron-like NSC-34 cells indicates DPRs are predominantly cleared by autophagy[154]. Loss of wildtype C9ORF72 function has also been identified in ALS/FTD caused by hexanucleotide repeat expanded C9ORF72[155]. A "double-hit" pathogenic mechanism has been proposed, given that wildtype C9ORF72 is suggested to participate in both autophagosome biogenesis and lysosome-dependent autophagic flux[149], whereby the hexanucleotide repeat expansion in C9ORF72 both generates DPR proteins and promotes neurotoxic DPR protein accumulation by impairing autophagy[156].

Cytosolic aggregates of the RNA and DNA binding protein TDP-43 are found in almost all patients ALS, as well as over 40% of patients with FTD[157]. While soluble (monomeric) TDP-43 is predominantly degraded by the ubiquitin-proteasome system, clearance of aggregated (oligomeric) TDP-43 requires autophagy[158,159]. Since TDP-43 depletion has been shown to downregulate autophagy by decreasing expression of the core autophagy protein ATG7[160], pathogenic feedback is again possible due to TDP-43 sequestration into aggregates causing loss-of-function.

Regarding other aggregate-prone proteins associated with ALS/FTD, autophagy is reported to degrade soluble and aggregated SOD-1, thereby preserving viability of mouse neuroblastoma cells expressing ALS/FTD-associated SOD-1 mutants[161]. FUS cytosolic inclusions are commonly decorated with the autophagy receptor p62[162]. However, interactions between autophagy and FUS-mediated ALS/FTD pathology are not well characterised. Finally, mitophagy (mitochondrial-selective autophagy) is known to clear defective mitochondria, which accumulate in ALS/FTD and compromise neuronal health[163].

Autophagy modulation as therapy in ALS/FTD

There are currently only two FDA-approved drugs for ALS (riluzole and edavarone), which have limited efficacy in slowing disease progression, and no FDA-approved drugs for FTD[164]. Autophagy modulation is therefore, an attractive therapeutic avenue in ALS/FTD [Table 2]. However, since defects in both autophagosome biogenesis and autophagic flux have been identified in ALS/FTD, autophagy inducers are clearly not panaceas for many forms of ALS/FTD.

A phase 2 clinical trial of rapamycin in ALS (RAP-ALS trial) has recently finished[165], with the results now awaited. This trial follows on from promising animal studies. For example, rapamycin treatment decreased TDP-43 inclusions and forebrain neurodegeneration in a FTD mouse model[166]. Increased autophagy was observed in the mouse forebrain after rapamycin treatment, with motor and behavioural phenotypes also ameliorated[166]. Rapamycin also reduced neuronal TBPH (TAR DNA binding protein 43 homolog) aggregates in a *Drosophila* ALS model, as well as partially rescuing lifespan and locomotive defects in these animals[167]. On the other hand, when studied using ALS/FTD models not based on TDP-43, rapamycin exhibited less therapeutic potential and sometimes worsened neurodegenerative phenotypes. Rapamycin accelerated motor neuron degeneration and shortened lifespan in a transgenic mouse model of ALS expressing disease-causing mutant SOD-1 (human G93A SOD-1), for instance[168]. This is suggested to relate to impaired autophagic flux in these animals, which is exacerbated by autophagy induction using rapamycin[168].

Experimental evidence concerning mTOR-independent autophagy inducers in ALS/FTD is similarly mixed. Treatment with trehalose ameliorated early motor phenotypes in the G93A SOD-1 transgenic mouse model of ALS, but failed to delay end-stage motor phenotypes and did not extend survival. This was despite

increased autophagy and reduced SOD-1 levels in the spinal cords of these animals after trehalose treatment[169]. Lithium showed initial therapeutic promise in patients with ALS[170]. However, subsequent higher powered clinical trials reported no benefit form lithium treatment on either disease progression or survival in patients with ALS[171-174]. The heterogeneous nature of ALS/FTD pathology, which extends to defects at multiple points in the autophagy pathway, therefore makes unlocking the therapeutic potential of autophagy modulation in ALS/FTD complicated, necessitating further study.

AUTOPHAGY AND AGEING

While the accumulation of protein aggregates is traditionally associated with neurodegenerative diseases, studies of post-mortem brains of aged individuals, who were not diagnosed with neurological conditions, shows the presence of amyloid plaques, neurofibrillary tangles, Lewy bodies, synaptic dystrophy and neuronal loss, consistent with the notion that the integrities of degradative pathways are challenged with age[175]. Indeed, there is increasing evidence suggesting that autophagy declines during ageing in many organisms and that this reduction plays a role in the functional deterioration of biological functions with age[176].

In multiple studies, autophagy gene transcripts decrease with age in the brain and muscle of *Drosophila*[177-179]. Transcriptional downregulation of *ATG5*, *ATG7* and *BECN1* with age was found in post-mortem human brains[180]. This correlates with the age-related decrease in autophagy proteins in the mouse hypothalamus[181] and human muscle[134], as well as lysosomal proteins in rat livers[182]. In a spatiotemporal analysis of autophagy in *C. elegans*, an age-dependent decrease in the numbers of autolysosomes and autophagosomes was observed in the intestines, muscles and neurons[183], corresponding with another study showing decreased autophagic activity in whole-body extracts of aged *C. elegans*[184]. Electron microscopy analyses have shown an accumulation of autophagic vacuoles and decreased ability to clear autophagic vesicles in mouse and rat livers with age[185-187].

Additionally, studies have shown a correlation between autophagy and lifespan. In *C. elegans*, decreased expression of orthologues of the mammalian *ATG1*, *ATG7*, *ATG12*, *BECN1* and *ATG18* lead to shortened lifespan[188]. Deletion of core autophagy genes, such as *Atg7* in flies, reduce life-span and cause accumulation of aggregated proteins in degenerating neurons[189], which was supported by findings revealing reduced lifespan in mutant flies with reduced *Atg1* and *Atg8* expression[179]. A study in senescence-accelerated mouse-prone 8 mice, a rodent model with accelerated ageing, showed an accumulation of autophagic vesicles in hippocampal neurons along with deficits in learning and memory with increasing age[190]. The age-dependent decline in autophagic function and lysosomal degradation was prevented with dietary restriction[191,192].

Overexpression of specific autophagy genes extends lifespan in flies and mice[179,193,194]. Additionally, overexpression of the TFEB orthologue, helix-loop-helix transcription factor *hlh-30* in *C. elegans* extended lifespan in an autophagy-dependent manner[195]. Disruption of the interaction of Beclin-1 with its negative regulator Bcl-2 achieved by introducing a point mutation in *BECN1(F121A)* in mice led to increased lifespan and decreased age-related renal and cardiac pathological changes and spontaneous tumorigenesis[196].

In addition to bulk autophagy, a decline in selective forms of autophagy, such as the autophagic degradation of mitochondria has been observed in aged worms, flies, mice and humans[37,197], possibly accounting for the increased presence of inefficient and toxic mitochondria that have been implicated in neurodegenerative and inflammatory pathologies[198]. In accordance with this notion, upregulation of mitophagy, as well as

pharmacological induction of mitophagy was found to improve longevity in worms[199-201].

It is evident that the progressive loss of the degradative capacity can lead to an accumulation of toxic proteins, as autophagy gradually decreases with age. As mentioned earlier, autophagic decline is especially relevant to neurons, as post-mitotic cells are unable to segregate dysfunctional proteins and organelles from daughter cells using mitosis, resulting in an increased reliance on autophagy. This can have a detrimental impact on neuronal health and may play a role in manifestation of neurodegenerative diseases.

Therapeutic potential of autophagy in ageing

In addition to therapeutic potential in neurodegenerative conditions, autophagy upregulation is also suggested to ameliorate phenotypes associated with normal ageing and extend healthy lifespan. Caloric restriction drives autophagy-dependent lifespan extension in both *S. cerevisiae* (budding yeast)[202,203] and *C. elegans*[204]. Similar results are reported using daf-2 mutant *C. elegans*, which exhibit lifespan extension due to defective insulin-like signalling dependant on autophagy[188,205]. Transgenic mice moderately overexpressing the core autophagy protein ATG5 in all tissues live 17% longer than wildtype controls and also exhibit "anti-ageing" phenotypes, such as enhanced insulin sensitivity[194]. Whether these phenotypes result directly from increased autophagy is not addressed in this study. More recently however, the lifespan extensions and milder age-related heart and kidney phenotypes exhibited by mice expressing constitutively active Beclin-1 (F121A mutant) has been shown to require increased flux through the autophagy pathway[196]. Pharmacological upregulation of autophagy has also been suggested to extend lifespan. For example, a multicentre trial has demonstrated that feeding aged (600 days old) mice rapamycin significantly increases age at 90% mortality[206]. This result has also been seen in *Drosophila* with rapamycin-induced lifespan extension dependent on expression of the core autophagy protein ATG5[207].

CONCLUSION

Although we can currently only speculate that autophagy induction would ameliorate age-related phenotypes in the brain and nervous system, neurodegeneration undeniably occurs predominantly against the backdrop of normal ageing. Accordingly, there is growing appreciation that research into normal ageing and age-related neurodegenerative diseases should be drawn closer together[128]. This includes incorporating ageing into models of neurodegenerative disease, which should increase model fidelity, leading to more efficient discovery of translatable disease-modifying therapies. Other considerations relevant to autophagy modulation as therapy in age-related neurodegenerative diseases include the mechanism through which autophagy is upregulated. The mTOR pathway for example, regulates numerous autophagy-independent processes such as cell growth and immunity[208]. Hence, autophagy inducers that function by inhibiting mTOR may have deleterious side effects. Variations in the primary disease-causing mechanisms exhibited by different individuals with the same neurodegenerative disease is another important consideration. Specifically, individuals with different variants of the same neurodegenerative disease may exhibit defects at different locations in the autophagy pathway. Another consideration is variations in pathology between cell types in the same individual at different stages of a neurodegenerative disease. It is also possible for autophagy defects to be of differential importance as a neurodegenerative disease progresses. Accordingly, an autophagy inducer might be beneficial in slowing the onset of a neurodegenerative condition, but not improve (or even worsen) end-stage symptoms. A nuanced approach that takes into consideration the complex relationship between autophagy, ageing and neurodegeneration is therefore required if the therapeutic potential of autophagy modulation is to be realised and produce new disease-modifying treatments for age-related neurodegenerative diseases.

DECLARATIONS

Authors' contributions

Contribute equally to the research and drafting of the article: Karabiyik C, Frake RA, Park SJ, Pavel M, Rubinsztein DC

Coordinate the first draft: Karabiyik C

Edite the article: Rubinsztein DC

Availability of data and materials

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Conflicts of interest

Rubinsztein DC is a consultant for Aladdin Healthcare Technologies SE, Drishti Discoveries, PAQ Therapeutics Inc. and Nido Biosciences. None of the other authors have any potential competing interests.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Ageing and Neurodegenerative Diseases

Review

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Diverse midbrain dopaminergic neuron subtypes and implications for complex clinical symptoms of Parkinson's disease

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Abstract

Parkinson's disease (PD), the most common degenerative movement disorder, is clinically manifested with various motor and non-motor symptoms. Degeneration of midbrain *substantia nigra pas compacta* (SNc) dopaminergic neurons (DANs) is generally attributed to the motor syndrome. The underlying neuronal mechanisms of non-motor syndrome are largely unexplored. Besides SNc, midbrain ventral tegmental area (VTA) DANs also produce and release dopamine and modulate movement, reward, motivation, and memory. Degeneration of VTA DANs also occurs in postmortem brains of PD patients, implying an involvement of VTA DANs in PD-associated non-motor symptoms. However, it remains to be established that there is a distinct segregation of different SNc and VTA DAN subtypes in regulating different motor and non-motor functions, and that different DAN subpopulations are differentially affected by normal ageing or PD. Traditionally, the distinction among different DAN subtypes was mainly based on the location of cell bodies and axon terminals. With the recent advance of single cell RNA sequencing technology, DANs can be readily classified based on unique gene expression profiles. A combination of specific anatomic and molecular markers shows great promise to facilitate the identification of DAN subpopulations corresponding to different behavior modules under normal and disease conditions. In this review,

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we first summarize the recent progress in characterizing genetically, anatomically, and functionally diverse midbrain DAN subtypes. Then, we provide perspectives on how the preclinical research on the connectivity and functionality of DAN subpopulations improves our current understanding of cell-type and circuit specific mechanisms of the disease, which could be critically informative for designing new mechanistic treatments.

Keywords: Parkinson's disease, ageing, dopaminergic neurons, dopamine, SNc, VTA, ALDH1A1, RNA sequencing, subpopulation

INTRODUCTION

Parkinson's disease (PD) is the second most common degenerative neurological disorder affecting millions of elderly individuals worldwide. PD patients display canonical motor symptoms, including resting tremor, slowed movement, impaired posture and balance, and rigid muscles[1]. The motor syndrome is generally regarded as the result of extensive loss of nigrostriatal dopaminergic neurons (DANs) in the *substantia nigra pars compacta* (SNc) of the midbrain[2,3]. Dopamine replacement medications and deep brain stimulation surgery can improve some of the patient's motor conditions. However, no cure is available. Moreover, long-term medication can cause severe side effects, such as dyskinesia and impulsive control disorders[4]. New mechanistic insights and therapeutic agents are still needed to improve patients' treatment and life quality.

In addition to motor symptoms, PD patients often suffer from depression, dementia, and other neuropsychiatric symptoms[5]. For example, PD patients often develop cognitive dysfunctions, which leads to Parkinson's disease dementia (PDD)[6,7]. Approximately 75% of PD patients develop dementia within 10 years of diagnosis, and the prevalence of PDD is 0.3%-0.5% in the general population older than 65 years[6]. The exact pathogenic mechanisms of PDD and other PD-related non-motor symptoms are largely unknown. The cognitive dysfunctions are not improved by levodopa, the most effective drug to treat the motor symptoms in PD[6]. Therefore, an important step in intervening a complex neurological disorder such as PD is to fully elucidate the functional roles of different neural circuits responsible for specific behavioral phenotypes.

It has been generally accepted that ageing, environmental toxins, and genetic mutations contribute to the etiopathogenesis of PD. Ageing is the most significant risk factor in the development of PD and other neurodegenerative diseases. Genomic instability, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, and altered intracellular communication are among the key ageing hallmarks[8]. Various environmental toxins and genetic risk factors have been linked to PD, which affect largely overlapping molecular and cellular pathways as those implicated in ageing[9-11]. However, the molecular genetic studies often fall short in pinpointing any specific cell-types or neural circuits critical for the alterations and impairments of motor and non-motor behaviors. To better understand how different brain cells and neural circuits control diverse behaviors will therefore provide the structural framework to better appreciate the impacts of ageing and environmental and genetic factors on the cause and progression of the disease-related behavioral abnormalities.

The midbrain DANs are composed of diverse neuron subpopulations based on the location of cell bodies, projection patterns, morphology, gene expression profiles, electrophysiological properties, physiological functions, and vulnerabilities to diseases[12-17]. Since a preferential degeneration of midbrain DANs represents the most significant neuropathological feature of PD, in this review, we focus our discussion mainly on the diversity of midbrain DANs in their distinct genetic makeups, connectivity, and functionality.

Because animal research has been instrumental in understanding the pathophysiological mechanisms and developing the treatments of PD, such as the current dopamine replacement therapy and deep brain stimulation surgery[18], we mainly summarize the research findings from preclinical animal models.

MOLECULAR GENETIC DIVERSITY OF MIDBRAIN DOPAMINERGIC NEURONS

Midbrain DANs, residing in the ventral region of the midbrain, are grouped together based on their ability to synthesize and release dopamine, a key neuromodulator involved in motor control and learning, motivation, cognition, and reward[19,20]. Dysfunction of midbrain DAN-mediated dopamine transmission has been associated with PD, schizophrenia, addiction, and other neurological and psychological disorders[5,21,22]. Traditionally, midbrain DANs can be divided into three main subgroups, retrorubral field (RRF, A8), SNc (A9), and ventral tegmental area (VTA, A10), in humans and rodents[23,24]. The midbrain DANs also differ in their axon projections to different brain regions and physiological functions. In addition, the SNc DANs are relatively more vulnerable to neuronal toxins in rodent PD models and preferentially degenerated in PD patients[25,26]. However, molecular genetic makers are needed to better characterize different DAN subtypes, as well as their distinctive connectivity and functionality.

The midbrain DANs selectively express genes critical for the dopamine synthesis, transport, and degradation, such as tyrosine hydrolase (TH), vesicular monoamine transporter 2 (VMAT2), dopamine transporter (DAT), and aldehyde dehydrogenase 1a1 (ALDH1A1)[27]. The midbrain DANs also express transcription factors critical for the DAN differentiation and survival, including nuclear receptor related 1 protein (NURR1), pituitary homeobox 3 (PITX3), and forkhead box protein A1/2 (FOXA1/2)[28,29]. The molecular genetic difference between SNc and VTA DANs was initially studied by immunostaining and *in situ* hybridization using preselected genetic makers. Those markers included G-protein activated inwardly rectifying potassium channel 2 (GIRK2/KCNJ6), which is more abundant in SNc DANs[30,31], and calbindin (D-28K, CALB1), which is more enriched in VTA DANs[32-34]. However, those genetic markers are not exclusively expressed by the SNc or VTA DANs. Additional genetic markers are required to specifically investigate the functions of different SNc and VTA DAN subtypes. The whole-genome gene expression studies using microarray and RNA sequencing (RNAseq) technology, especially the latest single cell RNAseq (scRNAseq) techniques, in combination with Laser Capture Microdissection (LCM) and Fluorescence-Activated Cell Sorting (FACS) procedures, provide the means to systematically identify distinct genetic identifiers for diverse DAN subpopulations[35].

Laser capture microdissection, fluorescence-activated cell sorting, and microarray studies of differential gene expression between SNc and VTA DANs

The TH-positive DANs in SNc and VTA were visually marked with a so-called rapid immunostaining procedure, and then collected separately by LCM. RNAs were extracted from the LCM-isolated SNc and VTA samples and subjected to microarray analyses. In both rat[36] and mouse[37] studies, numerous differentially expressed genes were found between SNc and VTA DANs. Some of those differentially expressed genes were reported by both studies. For example, the expression of Igf1, Gad1, Drd2, and Sncg is higher in the SNc, while the expression of Otx2, Tacr3, and Lpl is higher in the VTA. However, very few genetic markers can be used to distinguish between the SNc and VTA DANs, indicating that the molecularly defined DANs may not always be confined within the anatomical boundaries. In our studies, while we found that the majority of ALDH1A1-positive DANs are distributed in the ventral tier of SNc, a minority population is scattered in a broad region in the VTA[38,39]. A combination of two or more genetic markers would be required to identify a distinct subtype of DANs in SNc or VTA. On the other hand, since there were non-dopaminergic cells within the LCM samples, additional immunostaining and *in situ* hybridization experiments are needed to verify the expression of any gene of interest in the DANs. Furthermore, because of difficulty in physically isolating small DAN clusters by LCM, the distinct gene

expression profile of DAN subtypes can only be elucidated by the later FACS and scRNAseq techniques.

Multiple subtypes of midbrain DAN identified by single-cell RNA-sequencing

By employing the newly available scRNAseq technology, recently multiple studies have been performed to reveal the diverse gene expression profiles of midbrain DANs at single cell level[35]. These high-resolution gene expression studies demonstrate more complex gene expression patterns in individual midbrain DANs, identify more DAN subtypes with additional genetic markers, and improve our understanding of the genetic diversity of midbrain DANs[14,40-43]. Based on distinct gene expression patterns, midbrain DANs may constitute about 10 or even more subtypes. However, different numbers of DAN subtypes were reported from different studies, which often used different subtype names and proposed different genetic markers for clustering the subtypes. These differences reflect different technical approaches and classification criteria in their studies, while presenting a challenge to other researchers to consistently define the major subtypes of midbrain DANs[44]. We highlighted seven major midbrain DAN subtypes and listed their unique classification criteria based on the published mouse scRNAseq studies [Figure 1]. The heterogenous gene expression profiles of midbrain DANs revealed by scRNAseq and verified by the follow-up RNA scope *in situ* hybridization demonstrate the distribution of distinct subgroups of DANs in the developing and adult mouse midbrains. For example, the ALDH1A1-positive SNc DANs, which are mainly located in the ventral tier of SNc, express high levels of Sox6, Th, Dat, Aldh1a7, Lmo3, Anxa1, and Sncg, but not Otx2 and CALB1[40,43]. By contrast, the ALDH1A1-positive VTA DANs, which are found to be intermingled with other cell types in the VTA, express high levels of CALB1 and Vglut2, but not Sox6[14,17,40,43-46]. Therefore, based on the co-expression of Sox6 or CALB1, ALDH1A1-positive DANs can be assigned to SNc or VTA subregions in the midbrain [Figure 1]. Further in-depth analyses of distinct gene expression in ALDH1A1-positive SNc or VTA DANs may reveal additional molecularly and anatomically defined subtypes responsible for distinct physiological functions. To reliably interpret scRNAseq data, future studies need to improve the sensitivity in detecting low-level gene expression, as well as increase the numbers of cells collected in each experiment.

DIVERSE SNC DOPAMINERGIC NEURON SUBPOPULATIONS

The midbrain DAN system is the largest and most complex in primates compared to rodent species, with up to 600,000 TH-positive cells in humans compared to ~25,000 TH-positive cells in mice[47]. In rodents, about half of the TH-positive cells across A8, A9, and A10 are found within the substantia nigra (SN)[47]. In monkeys and humans, there is both a large increase in the number of TH-positive cells and an even larger percentage (> 70%) of the number of TH-positive cells that are located in the SN[47]. Beyond simply noting the number and percentage of SNc dopamine neurons across species, studies have also gone on to document the anatomic, molecular, and functional diversity of SNc DANs.

Anatomic and molecular diversity of SNc dopaminergic neuron subtypes

The DANs in the midbrain of rodents and primates can also be classified into a dorsal tier and a ventral tier[48-50]. The SN itself can be further subdivided into the SNc and *substantia nigra pars reticulata* (SNr). DANs are very densely packed within the SNc, while neurons are more sparse and diffuse within the SNr[51]. The DANs in the SNc are well known for their projections to the dorsal striatum (which consists of the caudate nucleus and the putamen) in what is known as the nigrostriatal dopamine pathway[52]. It has been well established that the striatum can be divided into two neurochemically distinct compartments, each with differential inputs, gene expression, connectivity, and distributions of neurotransmitters and neuromodulators[53-61]. These two compartments are referred to as striosomes (also known as patches) and matrix. When considering striatal input from midbrain DANs, unique sets of DANs produce projections to the patch and matrix compartments in both rodents and primates[62,63]. This organization of SNc DAN projection begins during development. Early on, dopamine input to postnatal striatum is organized into

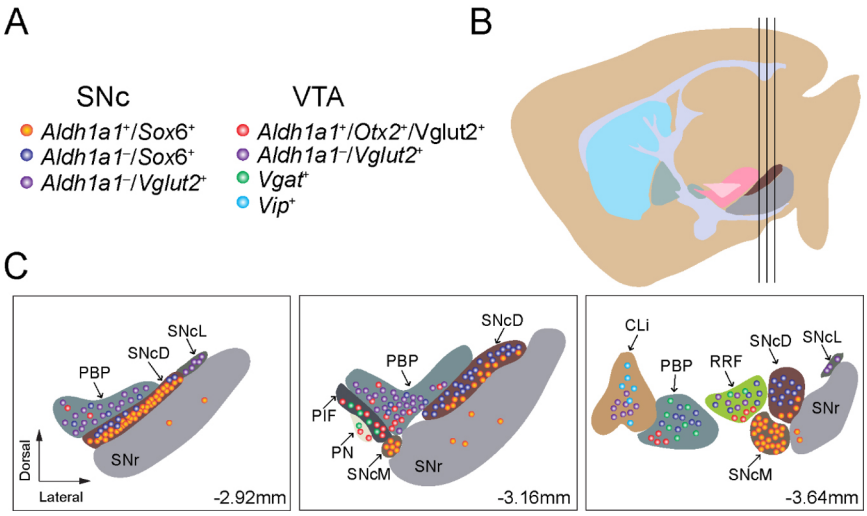

Figure 1. We outline the regional distribution of seven molecularly defined DAN subtypes in the SNc and VTA: (A) a list of molecularly defined DAN subtypes in the SNc and VTA; (B) a sagittal view of adult mouse brain where the three vertical black lines mark the positions of three cross-sections depicted in (C); and (C) regional distribution of molecularly defined DAN subtypes in the midbrain at Bregma-2.92, -3.16, and -3.64 mm. SNcD: SNc dorsal; SNcM: SNc medial; SNcL: SNc lateral; PBP: parabrachial pigmented nucleus; Cli: caudal linear nucleus of the raphe; PN: paranigral nucleus; PIF: parainterfascicular nucleus; SNc: *substantia nigra pars compacta*; VTA: ventral tegmental area.

patch compartments. As development progresses, the matrix is eventually innervated by its own DA afferents[50,52,64,65].

Recent work has demonstrated that even within a specific target region, DAN subtypes have partially overlapping yet distinct projections[66]. With respect to SNc DANs, three projection patterns were identified. These projections segregate along different axes of the caudate putamen. One of these projection patterns projects for the most part to locomotor areas of the striatum and consists of neurons positive for ALDH1A1, Sox6, and Ndnf that mainly project their fibers to rostral, intermediate, and caudal caudate putamen[66]. Another projection group originating in the dorsal SNc consists of neurons positive for CALB1 and Sox6 but negative for ALDH1A1 that project to the medial rostral caudate putamen and ventromedial regions of the intermediate and caudal caudate putamen. A third group consists of neurons positive for Vglut2 and CALB1 but negative for Sox6 that are located in the lateral SNc and project to the tail of the caudate putamen more so in response to novel cues and salience than error prediction[67]. DANs that project to cortical and limbic areas in both rat and monkey, while mostly derived from the VTA, are also present in lower numbers in the dorsal tier of the SNc[47,68-70].

In addition to projections, research has also investigated the inputs to nigrostriatal DANs and supports an integration of inputs from the autonomic, somatosensory, and motor areas. The greatest source of input is from the dorsal striatum, with a lot of input coming from the globus pallidus as well[71]. There are also notable projections from the central nucleus of the amygdala, entopeduncular nucleus, bed nucleus of stria terminalis, paraventricular hypothalamus nucleus, parasubthalamic nucleus, zona incerta, superior colliculus, supraoculomotor periaqueductal gray, dorsal raphe nucleus, pendunculotegmental nucleus, cuneiform nucleus, and parabrachial nucleus[71]. Additionally, strong excitatory inputs from the subthalamic nucleus and somatosensory and motor cortices may contribute to quick responses in the SNc DANs during salient events.

Functional diversity of SNc DAN subtypes

Various genetic, behavioral, and pharmacological studies have established a role of the nigrostriatal dopamine pathway in motor function, with reward largely associated with the mesolimbic dopamine pathway, which consists of dopaminergic connections that project from the VTA to the ventral striatum[49]. However, research also supports the importance of the nigrostriatal dopamine pathway in reward, suggesting that both SNc and VTA midbrain DANs have altered firing in response to reward prediction and prediction errors[72]. Studies show that reward-predicting stimuli result in SN activation, highlighting the fact that changes in midbrain DAN activity are not limited to the VTA[73]. When considering SNc DANs specifically, there is again support for neuronal activation in response to reward or sensory stimuli that predict reward and inhibition by aversive stimuli[74]. These DANs are located within the ventromedial part of the SNc adjacent to VTA, which raises the need to better define those two DAN subpopulations with more definitive genetic makers. On the other hand, there are also some SNc DANs that are activated by aversive stimuli or cues that predict aversive stimuli, which are located in the dorsolateral part of the SNc[74]. The role of dopamine is expanding further to include response to novel, salient, and even aversive stimuli[75-81].

The entire reward circuitry within a brain is a complex neuronal network with different aspects of reward- and incentive-based learning associated with pathways and connections within the larger reward network[49]. Reward systems are strongly associated with not only reward processing but also cognitive planning and motor control pathways that together all contribute to implementing an action plan for goal-directed behavior in response to reward and motivation[49]. For a long time, the basal ganglia were well known mostly for their role in motor behavior[49]. The nigrostriatal pathway has commonly been recognized as being involved in the facilitation and control of voluntary movement[82]. Nigrostriatal DANs allow information regarding movement to be sent from the SN to the striatum and are critical for normal movement capabilities. When information is transmitted to the striatum, desired movements can be initiated[83]. Nigrostriatal dopamine input to the striatum also plays a role in the initial learning and memory of sequential motor tasks and motor skill learning[84,85]. We now know that the basal ganglia are involved not just in motor function, but more widely in a range of emotional, cognitive, and motivation functions that allow for goal-directed behaviors[49].

In terms of where in the SNc motivational value or salience signals, reward value coding, activity in response to aversive stimuli, and signals for trial start of unexpected time cues occur, each specific signal type is most strongly associated with some part within the SNc[75]. Ventromedial SNc DANs are strongly associated with motivational value signals, dorsolateral SNc DANs are strongly associated with motivational salience signals, ventromedial SNc DANs are strongly associated with standard reward value coding, lateral SNc DANs are strongly associated with aversive cues that lead to excitation, and DANs throughout the SNc are associated with trial start cues and unexpected time cues[74,86,87]. Adding to what we know about reward prediction error of dopamine signaling, recent research has worked on investigating the role of dopamine in impulsivity[88-91]. Different SNc groups and their corresponding projections are suggested to be able to distinguish between decisional impulsivity and motor impulsivity. Research suggests that the two types of impulsivity are regulated by different dopamine systems[88]. Specifically, the medial SNc dopamine group plays a role in value-coding, or the difference in response between reward and punishment, and is associated with impulsive choice. On the other hand, the ventral and lateral SNc DAN groups play a role in salience-coding, or the difference in response between either reward or punishment and aversive stimuli, which are associated with response inhibition.

Differences in function between ventrally and laterally positioned SNc DANs have also been demonstrated for other behaviors. ATP-sensitive potassium (K-ATP) channel activity in medial SN DANs, but not lateral SN DANs, allows for *in vivo* burst firing that is critical for novelty-dependent exploratory behavior but not standard locomotion[92]. Research also suggests nigrostriatal DAN activity signals the start or stop of action sequences and is involved in action selection, in addition to its role in reward-based learning[93-96]. Pathways that involve the SNc are also critical for learning to orient to food cues and for increasing motivation to perform reward-seeking actions[75,97-101].

Distinct characteristics of ALDH1A1-positive SNc DAN subpopulation

Although the classification of midbrain DANs into the A8, A9, and A10 cell groups is still commonly used, recent work suggests the existence and importance of functional and gene expression heterogeneity of subgroups within each of these cell groups[12,14,66,75,102-105]. One subtype of SNc DANs that is of particular interest, especially in the context of PD, is the ALDH1A1-positive subtype of SNc DANs[106]. ALDH1A1 oxidizes the highly reactive dopamine catabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL)[107], and neurons that express ALDH1A1 correspond to ventral tier nigrostriatal DANs, which are preferentially degenerated in PD[15,38,108]. The ALDH1A1-positive SNc DAN subtype accounts for about 70% of SNc DANs[15,39]. The ALDH1A1-positive subtype has its own pattern of projections and inputs that is distinct from the connectivity patterns of other SNc DAN subtypes, in addition to playing an important role in the acquisition of skilled movements in rodent models of PD[39,106].

ALDH1A1-positive DANs in the SNc project primarily to the dorsal striatum[39,109]. The projections to the striatum appear to be arranged along a medial to lateral axis based on the position of their cell bodies. The more caudal ALDH1A1-positive SNc DANs project to more rostral areas in the striatum[39]. However, only a small fraction of ALDH1A1-positive SNc DAN axons converge to the striosomes[39,66,110]. In terms of inputs, ALDH1A1-positive SNc DANs receive most of their input from the caudate putamen, but they also receive substantial input from other areas in the striatum, pallidum, hypothalamus, and midbrain [Figure 2A][39]. While there is less input from cortical areas, most of the input that is derived from there is coming from primary and secondary motor cortices and the somatosensory cortex, implicating a role of the regulation of ALDH1A1-positive SNc DANs in sensorimotor activity[39]. Relative to ALDH1A1-positive DANs in the VTA, those in the SNc receive more inputs from the caudate putamen, particularly the lateral caudate putamen which is heavily innervated by the ALDH1A1-positive SNc DANs, supporting a strong reciprocal innervation[39]. Compared to inputs to all SNc DANs, more inputs to ALDH1A1-positive SNc DANs come from the ventral striatum and the hypothalamus while fewer inputs originate from neurons in the cerebral cortex, pallidum, amygdala, and midbrain[39,71].

With respect to its distinct functional role, ALDH1A1-positive SNc DANs are critical for the acquisition of motor skill learning in the rotarod task in a mouse model that is not alleviated by dopamine replacement therapy[39]. More generally, it seems that timely and dynamic regulation of dopamine release by ALDH1A1-positive SNc DANs plays an important role in mediating goal-oriented actions requiring high levels of motor motivation. Compared to the ALDH1A1-negative SNc DANs, the ALDH1A1-positive SNc DANs possess a distinct rebound activity after hyperpolarization [Figure 2B], resulting in alteration of firing pattern from evenly paced tonic firing to high frequency burst firing[16,106,111]. The burst firing may lead to increase of dopamine release, an indicator for engagement of certain actions. The presynaptic inhibitory inputs from striosome direct pathway spiny neurons play a major role in regulating the transition from tonic firing to burst firing of ALDH1A1-positive SNc DANs[111]. During the burst firing, the cytosolic dopamine can be oxidized by mitochondria-attached monoamine oxidase (MAO) to produce H_2O_2 and DOPAL [Figure 2C][112]. The H_2O_2 can then be utilized for mitochondrial Complex IV-mediated ATP production[112], while the increase of ATP production may lead to increased dopamine release and reuptake,

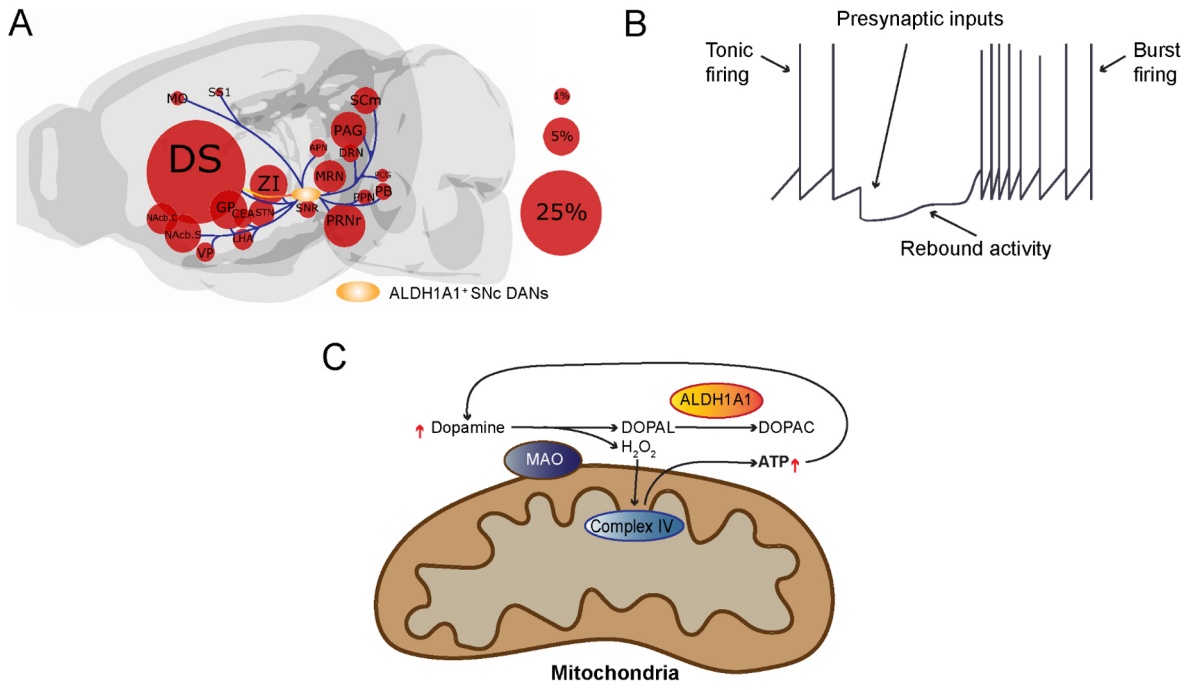

Figure 2. Presynaptic inputs alter the firing pattern of ALDH1A1-positive SNc DANs, in which the burst firing recruits dopamine for ATP production: (A) We outline the locations of major input neurons in the brain that directly innervate ALDH1A1-positive SNc DANs. While the ALDH1A1-positive SNc DANs integrate diverse synaptic inputs from different brain regions, they provide the output mainly to the dorsal striatum. The size of the circles represents the percentage of overall inputs. (B) Presynaptic inputs regulate the transition from tonic firing to burst firing of ALDH1A1-positive SNc DANs. (C) Cytosolic dopamine can be oxidized by mitochondria-attached MAO to produce H_2O_2 and DOPAL. H_2O_2 can be used for Complex IV-mediated ATP production, while the cytotoxic byproduct DOPAL can be neutralized by ALDH1A1. The increase of ATP production may lead to increased dopamine release and reuptake, resulting in further increase of ATP production and dopamine release during the burst firing. DS: Dorsal striatum; ZI: zona incerta; NAcb.C: nucleus accumbens core; NAcb.S: nucleus accumbens shell; MO: motor cortex; SS1: somatosensory cortex; VP: ventral pallidum; GPe: globus pallidus; STN: subthalamic nucleus; PAG: periaqueductal gray; APN: anterior pretectal nucleus; MRN: medial raphe nucleus; PRNr: pontine reticular nucleus roastral; PPN: posterior pretectal nucleus; PCG: pontine central gray; SCm: superior colliculus medial; DRN: dorsal raphe nucleus; CEA: central nucleus of the amygdala; LHA: lateral hypothalamus; PB: pontine parabrachial nucleus.

resulting in further increase of ATP production and dopamine release during the burst firing [Figure 2C]. The presence of ALDH1A1 neutralizes the cytotoxic byproduct DOPAL and maintains the normal function and survival of this distinct DAN subtype[106]. However, there is still more research needed regarding the integration of specific excitatory and/or inhibitory inputs in functionally regulating ALDH1A1-positive SNc DANs before we fully understand the role of ALDH1A1-positive SNc DANs in motor learning.

DIVERSE VTA DOPAMINERGIC NEURON SUBPOPULATIONS

VTA holds an intriguing and diverse population of DANs in the midbrain[104]. The VTA neurons synthesize several major neurotransmitters, including dopamine, GABA, and glutamate[113]. While most neurons in the VTA are dopaminergic, the exact percentage can vary between subregions. Overall, only around 50% exclusively secrete dopamine, while others co-secrete glutamate and GABA or do not secrete dopamine at all[114,115]. In contrast to the SNc, which is greatly associated with movement, the VTA is more related to emotion and cognition[116,117]. All of these functions are impaired in PD[118], making both regions of great translational interest. The anatomical separation of the VTA is not clear, hence its name ending in "area", not "nucleus". Its separation from the SNc is best described based on both its functional projections and molecular markers[104]. While the SNc tends to project to the striatum via the nigrostriatal pathway, which is critical for motor movement, the VTA largely mediates dopamine secretion through limbic and cortical

projections[119]. These two, divergent VTA pathways are known as the mesolimbic and mesocortical, respectively[120]. They are especially important for incentive-based behavior, motivation, and cognition[121-123]. The mesocortical pathway projects to the prefrontal cortex and is related to the attention to reward experience, interpretation of motivation, and the cognitive appraisal to seek out reward again[122]. On the other hand, the mesolimbic pathway projects to limbic structures, such as the amygdala, nucleus accumbens, and hippocampus[124]. Different stimuli can lead to different degrees of dopamine secretion or firing patterns[125-127], helping to explain why some drugs may be more addictive than others.

Molecular, anatomical, and functional subclassification of VTA components

The VTA DANs were originally classified into five subgroups but are presently further segregated into seven due to analyzing the differential expression of GIRK2, calbindin, DAT, and TH[128]: interfascicular nucleus (IF), rostral linear nucleus (RL), caudal linear nucleus of the raphe (CLi), paranigral nucleus (PN), parabrachial pigmented nucleus (PBP), parainterfascicular nucleus (PIF), and ventral tegmental area rostral (VTAR) [Figure 1]. The VTA has a greater diversity of DAN subtypes when compared to RRF and SNc groups. Despite these classifications, several studies have detected heterogeneity, even within the clusters, both molecularly and functionally[129]. Some of the main differences found were between the medial and lateral VTA DANs[128,130]. These regions tended to have more calbindin-positive DANs and less DAT- and GIRK2-positive expression; in addition, the IF subregion had the smallest size DANs out of all VTA nuclei[128]. Moreover, in the lateral VTA, PBP and VTAR are on the edge of the SNc but remain distinctly classified as VTA neurons based on molecular markers[128]. Compared to SNc neurons, the PBP has a greater ratio of calbindin/TH-positive neurons, with neurons immersed in fibers aligned in different directions[128]. Likewise, VTAR DANs are remarkably less densely packed, making them distinct from the SNc[128]. There have been attempts to better organize midbrain DAN clusters, such as with single cell expression profiling in neonatal brains[129]. Certain genes were enriched in the SNc or the VTA and were used to analyze their relative expression across clusters[129]. For instance, Otx2 is mostly expressed in VTA region, while SOX6 is mostly expressed in SNc[129]. Moreover, ALDH1A1 served as a useful distinguishing marker between clusters, related to how ALDH1A1 is anatomically mainly expressed in the ventral VTA and SNc[129].

Much remains unknown about output projection patterns from the VTA, especially with regards to different DAN subpopulations[131]. An exception is the RLi subregion, which has been well studied with regards to outputs[132]. This is further complicated by the presence of co-secreting neurons, which may secrete any combination of dopamine, GABA, and glutamate[133]. Even more, certain neurons do not secrete or reuptake dopamine despite synthesizing it and expressing TH[104]. The different circuits and functions of these VTA DAN subtypes is still somewhat a mystery and is motivating further research on projection patterns. Furthermore, function and regulation of VTA DANs can be influenced by differential upstream groups of neurons to yield reward and aversion[134,135]. This highlights how complex inputs can be in regulating the already complex diversity of VTA DANs. Better determining their transcriptome, connectivity, and functionality may be useful in better classifying and understanding the very diverse populations of DANs in the VTA.

DIVERSE SUSCEPTIBILITY OF DOPAMINERGIC NEURON SUBTYPES IN PARKINSON'S DISEASE

PD involves loss of both SNc and VTA DANs[136]; however, the contribution of DAN loss in the VTA to PD symptoms remains controversial among scientists and physicians alike. Results from a series of studies comparing DAN counts in SNc and VTA across PD and healthy control brains stained with TH demonstrate the involvement of the VTA in PD[136]. Although researchers observed significantly more degeneration of DANs within the SNc, the substantial neurodegeneration within the VTA may contribute

to PD-related clinical symptoms, especially the non-motor syndrome[136]. It needs to be pointed out that in the previous studies the boundary between SNc and VTA was often drawn arbitrarily based on a few anatomical landmarks [Figure 1]. With the availability of increasing numbers of distinctive genetic markers, the function and survival of SNc and VTA DAN subtypes will be investigated in a more precise and molecularly defined way. In other words, future experiments are expected to pinpoint which DAN subtypes in the SNc and VTA DANs are involved, and the extent to which they are degenerated in PD. We suspect that the selective susceptibility of DAN subtypes during the progression of PD may contribute to the complex clinical manifestations of the disease.

Diverse vulnerability of SNc DAN subtypes

A major pathological characteristic of PD, the major cause of parkinsonism, is the preferent neurodegeneration of SNc DANs. The loss of SNc DANs is associated with both bradykinesia and rigidity, two of the major motor symptoms that occur in PD[137]. As for the reason the SNc DANs are preferentially degenerated in PD, there are multiple distinct characteristics pertaining to endogenous neurotransmitter expression, structure, physiology, and local environmental conditions of the SNc DANs that may make them intrinsically vulnerable to degeneration[138].

Dopamine as a neurotransmitter

The fact that these neurons are dopaminergic suggests that dopamine itself might play a role in contributing to selective vulnerability[139]. In many ways, the oxidative chemistry of dopamine can be associated with mechanisms underlying pathways involved with dysfunction of protein degradation, deficits in mitochondria processes, protein aggregation, neuroinflammation, and oxidative stress[139,140]. Free radicals and quinones that ultimately derive from the presence of dopamine can go on to interact with different cellular components and eventually contribute to the pathogenesis of PD. In the cytosol, dopamine is synthesized from tyrosine. Specifically, tyrosine is converted into L-dihydroxyphenylalanine (L-DOPA) by TH, the rate-limiting enzyme in dopamine synthesis, which is subsequently converted into dopamine by aromatic amino acid decarboxylase[138,139]. Dopamine is stabilized by the low pH within synaptic vesicles following its sequestration there by VMAT2[141]. Unlike when dopamine is within vesicles, it readily self-oxidizes in the cytosol, and too much oxidized dopamine is thought to be toxic to the DANs[138]. One consequence of oxidation is the formation of reactive oxygen species (ROS), which, when present at too high of levels, can damage DNA, RNA, proteins, and lipids[142]. Dopamine is also capable of being oxidized into reactive quinones (DAQs) that may also alter DNA, proteins, and lipids or form DNA-adducts leading to DNA damage responses[143,144]. Self-oxidation of dopamine within the neurons is supported by the presence of neuromelanin, as DAQs serve as precursors for two different portions of neuromelanin: pheomelanin (the polymeric core of neuromelanin) and eumelanin (the polymeric surface of neuromelanin)[145,146]. Although neuromelanin is believed to be non-toxic and maybe even neuroprotective[147-149], neurons in the SNc with high levels of neuromelanin ultimately have the greatest vulnerability in PD[25,150]. This is consistent with the observation that neuromelanin can increase α-synuclein levels via inhibition of proteasomal degradation which ultimately contributes to Lewy body pathology[145]. α-synuclein is natively unfolded and will associate with vesicle membranes. It can also form into oligomers, also known as protofibrils, which are able to permeabilize dopamine-containing synaptic vesicles and cause dopamine to leak into the cytosol[151]. The cytosolic dopamine can then react with α-synuclein and create an adduct that slows the conversion of photofibrils into fibrils. This effectively maintains the presence of photofibrils, which causes more synaptic vesicle permeabilization and thus more dopamine leakage, creating a cycle of increasing cytosolic dopamine[152-154]. α-synuclein-dopamine adducts can also block chaperone-mediated autophagy by preventing lysosome receptors from accepting proteins and breaking them down, contributing to an increasing number of toxic proteins in the neuron[155].

Dopamine in the cytosol can also be metabolized by MAO to form DOPAL, which is very toxic and can enhance α-synuclein aggregation[156]. To protect itself, neurons will attempt to condense oxidized products, turning them into neuromelanin[147]. MAO, in its metabolization of dopamine, has been thought to produce hydrogen peroxide in the cytosol[112,157]. Recent evidence in both mouse and human DANs, however, shows that dopamine metabolism by MAO does not increase hydrogen peroxide levels in the cytosol; instead, it increases mitochondrial electron transport chain activity[112]. Perhaps electrons generated from dopamine metabolism are not transferred to oxygen but rather brought through the mitochondrial intermembrane space to the electron transport chain[112]. Although there is certainly a lot of evidence suggesting a potential pathogenic role of dopamine in the SNc neurons, there are other neurons that do not express dopamine but are still degenerated to some degree in PD. There are also DANs in other areas of the brain that are not degenerated in PD[138]. This suggests that dopamine's presence alone in SNc DANs is not the only factor contributing to the neurons' intrinsic vulnerability.

Preferential degeneration of ALDH1A1-positive SNc DANs in PD

DANs in the ventral tier of SNc displayed the most profound loss in the postmortem brains of PD patients[15,158]. These ventral SNc DANs selectively express ALDH1A1[15,38]. As one of the 19 members of ALDH superfamily genes in the human genome[159], ALDH1A1 is the only one exclusively expressed by the midbrain DANs[15,38]. Within DANs, ALDH1A1 converts the highly reactive dopamine catabolic intermediate cytotoxic DOPAL into a less toxic acid form and thereby protects DANs against DOPAL-induced cytotoxicity[160]. The generation of DOPAL used to be regarded as a passive event due to the oxidation of dopamine leaked in the cytosol[38,161]. A recent study suggests that the oxidation of cytosolic dopamine may actively participate oxidative phosphorylation and ATP production in DANs in response to intensive extracellular stimulations[112]. ALDH1A1 would be a key enzyme to neutralize the production of cytotoxic DOPAL during this process. The levels of ALDH1A1 expression are downregulated in PD[15,162], while genetic ablation of ALDH1A1 and ALDH2 causes robust SNc DAN loss and motor impairments in aged mice[163]. Genetic variants in the ALDH1A1 gene locus were associated with sporadic PD cases[164], while epidemiological studies link high exposure of fungicide benomyl, a potent ADLH inhibitor, to increased PD risk[165]. The reduction of ALDH1A1 expression may render the ventral SNc DANs more susceptible to cytosolic stresses[38]. ALDH1A1 expression level and activity can be used as an important biomarker to monitor the progression of the disease, while enhancement of ALDH1A1 activity could serve as a potential therapeutic strategy[38].

Distinct neuronal architecture: long axonal arbor and lots of branches

The terminal field of SNc DANs are thought to be lost before cell bodies based on observations of human brains, suggesting this part of the SNc DANs is most vulnerable[158,166-169]. Multiple structural characteristics of the SNc DANs, specifically their terminal fields, may contribute to the selective vulnerability. The terminal fields of the SNc DANs are very large, dense, and wide and thus form a uniquely large number of synapses with the striatum and have many sites where neurotransmitter release can occur[170-174]. Additionally, the SNc DAN axons are long and unmyelinated with complex axonal arborization[174]. This creates a high energetic burden for action potential propagation which might contribute to increased basal stress on these neurons and make them more vulnerable to further environmental and genetic stressors[172,174,175]. Aside from long axons possibly contributing to high bioenergy requirements, the many dopamine release sites with their need for large vesicle pool and release machinery such as α-synuclein could also be predicted to be relatively energetically expensive[138].

The energetically expensive physical maintenance of protein synthesis, cytoskeleton structure, membrane potential, and synaptic transmission of such a complex neuronal architecture as that of SNc DANs may also

increase vulnerability[138]. Although high, the neurons are normally able to handle the energetic demand. However, it is when exposure to any of multiple possible environmental or genetic perturbations disrupt the link between energy production and demand that problems begin to occur[174]. All the necessary proteins, lipids, and organelles that are in the soma must be transported to distal sites via anterograde transport while some damaged structures need to be brought back to the soma for degradation via retrograde transport[176-180]. Importantly, all the transporting is happening within one long axon, which means the transported materials are much more likely to get crowded and experience disrupted trafficking[177,178]. Disrupted transport of mitochondria particularly would severely limit the spatial distribution of mitochondria, which serve as the main energy source of neurons, and thus the spatial distribution of energy sources within the neuron. Ultimately, it seems that the long and highly branched axons of SNc DANs are particularly susceptible to the disruption of mitochondria dynamics, making these neurons particularly prone to axon degeneration.

Autonomous firing

Beyond distinct structural properties, SNc DANs also exhibit unique physiological characteristics that may contribute to increased and selective vulnerability to degeneration. One such characteristic is that the neurons are slow, autonomous pacemakers (unlike most neurons in the brain) with broad action potentials due to Ca^{2+} influx[181]. This means that the SNc DANs will spike, resulting in Ca^{2+} entry into the cell, despite receiving no excitatory input[182-188]. Autonomous firing and subsequent Ca^{2+} entry into the neuron create an energy demand. Additionally, the continuous firing and broad action potentials cause the ionic gradients underlying excitability to be eliminated[138]. To maintain an electrochemical gradient, ATP-dependent pumps must be maintained, contributing further to energy demand. The neurons will also respond to synaptic inputs which can trigger burst firing and further increase Ca^{2+} loading within the neurons[189,190].

SNc DANs express low-threshold variants of the CaV1.3 L-type calcium channel, unlike most other pacemaker neurons. There is a sustained Ca^{2+} influx because these channels never fully close[186,187]. Ca^{2+} in the cytosol easily crosses the outer membrane of mitochondria via large nonselective pores; crossing the inner membrane of the mitochondria, however, is tightly regulated. The mitochondrial Ca^{2+} uniporter is a selective ion channel that allows Ca^{2+} to enter the matrix[191,192]. Ca^{2+} within the mitochondria increases tricarboxylic acid enzyme activity and oxidative phosphorylation[193-195] which will eventually lead to ROS[140,181]. Any generated oxidants that are not taken care of by antioxidant defenses can cause a continuous oxidative stress in the mitochondria[196,197]. Continuous oxidative stress in the mitochondria can have many negative effects, including increased sensitivity of SNc DANs to toxins and ageing[181,198,199].

Sustained levels of Ca^{2+} that remain in the cytosol can also have negative effects, specifically by leading to increased α-synuclein accumulation[200-202]. The high levels of Ca^{2+} in the SNc DANs is not buffered much by calbindin[203]. Without sufficient buffering, Ca^{2+} can diffuse away to various targets within the cell. Mutant α-synuclein is able to increase pacemaker activity (and the subsequent stress it causes) by disrupting A-type K^+ channel[204]. Supporting the intrinsic vulnerability of the SNc DANs, this occurs in SNc but not VTA DANs.

Synaptic partners

The unique cellular environment and local neurons present near the SNc DANs may also contribute to their distinct vulnerability to neurodegeneration. Research now supports the concept that SNc DANs are relatively depolarized under normal, healthy conditions. The membrane potential of the neurons usually sits between -60 and -45 mV[205,206]. At this potential, the Mg^{2+} block of N-Methyl-D-aspartate receptors (NMDARs) is relatively weak. Additionally, for the most part, SNc DANs express NMDARs containing the GluN2D subunit, which is relatively insensitive to Mg^{2+}[206,207]. Together these observations suggest that there

are lots of NMDAR openings at any given time, even in healthy neurons, which can increase Ca^{2+} loading and oxidant stress in the SNc DANs. Upon prolonged exposure to high levels of glutamate outside the SNc DANs, metabotropic glutamate receptors (mGluRs) are also activated. Via a process referred to as Ca^{2+}-induced Ca^{2+} release, activation of both mGluRs and NMDARs prompt the endoplasmic reticulum to release Ca^{2+}[208].

Additionally, the SNc DANs are surrounded by a relatively high density of microglia, which are involved in inflammatory responses[209]. Microglia can become activated when exposed to proinflammatory molecules, toxins, and protein aggregates, prompting the microglia to then release molecules that may be harmful to the neurons[210-213].

Involvement of VTA DAN subtypes in PD-related non-motor symptoms

Abnormal prefrontal dopaminergic and cholinergic circuits lead to a variety of cognitive symptoms, including executive dysfunction, hallucinations, and psychosis[214]. Further, the development of cognitive symptoms may serve as a predictor for PDD and amnestic dysfunction as the disease progresses[214]. To examine the neurophysiological underpinnings of PD-related depression, researchers lesioned both the SNc and VTA of rats and measured the effect of L-DOPA and citalopram administration, an amino acid precursor to dopamine and a selective serotonin reuptake inhibitor, respectively[215]. While depressive-like behavior was induced by lesioning either the SNc or VTA, symptoms were alleviated by either drug treatment[215]. These results suggest a link between both SNc and VTA DAN deficits with PD-related depression, as well as the involvement of serotonergic pathways[215]. Another study found that partial bilateral ablation of the SNc results in both motor and non-motor symptoms, while ablation of both the SNc and the tail of the VTA relieves symptoms in PD mouse models[216]. These data demonstrate the compensatory role of the VTA in moderating the DA system in response to SNc neuronal ablation[216]. Although researchers tend to focus on the involvement of the SNc due to severe neurodegeneration of DANs localized in this brain region, these findings support the role of the VTA in PD symptoms[217].

VTA DAN subpopulations in the formation of declarative memory and PDD

Accumulative evidence supports an association of dopaminergic dysfunction with PDD[117,218]. PDD is likely resulted from extensive degeneration of midbrain DANs beyond the SNc regions in the late stages of PD. Which subpopulations of midbrain DANs contribute to PDD remains to be determined. With the advancement of gene profiling in individual neurons, many genetically defined DAN subtypes have been identified in different SNc and VTA subregions[14,17,43]. Using an intersectional genetic labeling strategy, a recent study found that a cluster of vesicular glutamate transporter 2-positive ($VGLT2^+$) DANs in the ventral VTA project predominantly to the entorhinal (ENT) and prefrontal cortices[66]. Interestingly, ENT atrophy is particularly associated with PDD[219]. By contrast, VTA DANs only sparsely project to the hippocampal formation[66]. Instead, the hippocampus receives the most dopamine inputs from the afferent fibers of locus coeruleus[220]. Therefore, it would be interesting to investigate the synaptic inputs and physiological functions of VTA-$VGLT2^+$ DAN subpopulations in declarative memory formation. The knowledge gained from this study will provide cell type and circuit specific mechanisms of PDD and lay the foundation for designing new therapeutic interventions for treatment of cognitive impairments in PDD.

VTA DAN subpopulations in temporal control of movement and PD

Past work indicates the role of VTA in temporal control, or "guiding movements in time to achieve behavioral goals"[221], as well as temporal expectation, or "the ability to anticipate when a stimulus occurs in time"[222]. Researchers experimentally manipulated prefrontal dopamine transmission from the VTA of rodents to examine the effect on temporal control during a fixed-interval task[221]. Temporal control was

impaired by viral RNA interference with VTA DA transmission, antagonists blocking dopamine receptor D1 (DRD1) in the medial prefrontal cortex, and optogenetic inhibition of prefrontal DRD1-positive neurons[221]. Further, temporal control during the fixed-interval task was improved by optogenetic stimulation of prefrontal DRD1-positive neurons[221]. These results suggest the involvement of mesocortical DAN projections and DRD1 within prefrontal cortex over temporal control of movement[221]. Another study investigated temporal expectation during a reaction time task in dopamine-depleted rats, while inhibiting DAN projections from the VTA with a selective neurotoxin[222]. Although VTA dopamine depletion did not alter movement and learning during the reaction time task, rats did not exhibit delay-dependent speeding[222]. These data suggest the involvement of mesocortical DAN circuits in temporal expectation[222]. Researchers also found that delay-dependent speeding was reduced by DRD1 antagonist but not DRD2 antagonists, indicating the role of prefrontal cortex DRD1 in temporal expectation[222]. Taken together, these studies suggest that VTA function significantly impacts temporal control and expectation, specifically highlighting the role of DRD1 subtype in animal models of PD.

DIVERSITY OF MIDBRAIN DOPAMINERGIC NEURON SUBTYPES IN NORMAL AGEING

Normal ageing is associated with minor neurodegeneration of DANs within the SNc, accompanied by a significant decline in voluntary motor control[223]. Brain imaging studies indicate a significant loss of DAN function due to normal ageing, linking deficits in DA neurotransmission to cognitive dysfunction[224]. Although dopamine neurotransmission may impact cognitive performance directly, it may also act in an age-dependent manner[224]. Normal ageing has been associated with impaired episodic memory, processing speed, and executive functioning[224]. While DAN degeneration is experienced in both PD and normal ageing, loss of DAN function occurs at a more rapid rate in PD relative to healthy elderly subjects[225]. Despite differential changes in the SNc, links between DAN degeneration and cognitive dysfunction are common across both groups[225]. Further, research suggests that gonadal hormones modulate DA pathways, with sex differences in DANs and disease progression of PD and dementia[226]. Researchers examined the effect of estrogen on SNc DANs in African green monkeys, finding that 30 days of estrogen deprivation led to permanent loss of more than 30% of DANs within the SNs[226]. Subsequent estrogen replacement only restored TH-immunoreactive cells when given 10 days after ovariotomy, but not 30 days after[226]. Taken together, research indicates demographic factors that may influence the degree and rate of cognitive decline experienced in PD, including both age and sex.

Past work suggests that dopamine plays a significant role in motivated behavior, such that DANs and cognitive function simultaneously decline in an age-dependent manner[227]. L-DOPA treatment has successfully enhanced reinforcement learning in elderly subjects, suggesting a link between DAN activity and motivated behavior in both PD and normal ageing[227]. Ageing also affects the progression of PD, including the age of symptom onset as well as the form and severity of PDD[228]. One longitudinal study found that the average PD patient experiences rapid DAN degeneration within the midbrain and progressive accumulation of Lewy bodies, which eventually invade the neocortex and cause PDD[228]. However, late-onset PD patients experience greater levels of Lewy bodies containing α-synuclein in addition to plaque formation, having a shorter disease course[228]. These data indicate how normal ageing may modulate the relationship between the DAN degeneration and decline in cognitive function.

Ageing-related mitochondrial dysfunction in DANs

Mitochondrial dysfunction may play a role in the age-dependent mechanisms underlying DAN deficits in the SNc, such that mitochondrial DNA mutations increase with age[223]. For example, human studies indicate high levels of deletions in mitochondrial DNA of both PD and healthy elderly subjects, linking mitochondrial dysfunction with number of deletions[223]. Researchers also found a 20% increase in

mitochondrial DNA deletions in old mice relative to young mice, also working in an age-dependent manner[223].

Ageing-related nitrative stress in DANs

Ageing serves as the highest risk factor for the development of PD, with nitrative stress potentially contributing to degeneration of the DA system[229]. Researchers assessed the link between nitrative damage and DAT levels in rhesus monkeys over time[229]. The number of DANs that underwent nitrative damage significantly increased with ageing in the SNc but not in the VTA. Further, the percentage of DANs that underwent nitrative damage was significantly higher in the SNc relative to the VTA[229]. These results demonstrate the age-dependent accumulation of nitrative damage and its role in selective neurodegeneration of the SNc DA system[229].

Ageing-related dysfunction of dopamine reuptake in DANs

DAT mediates the reuptake of dopamine from extracellular space into DANs[230]. One study assessed whether a decline in DAT expression was responsible for functional differences in DAT[231]. Although DAT immunoreactivity within the striatum, SNc, and VTA was not altered in an age-dependent manner, a 60% decrease of VTA TH was recorded only in older rats[231]. Further, a 30% decrease in dopamine reuptake and DAT protein recovery was recorded in the striatal synaptosomes of old rats relative to young rats[231]. These results indicate that reduced DAT expression on the plasma membrane results in age-related decline in DAT function[231]. Taken together, past work has established a robust relationship between ageing and DAT function in animal models of PD.

Positron emission tomography has been used to compare DAT levels across PD and healthy control subjects over time[232]. One longitudinal study found lower baseline DAT expression in PD relative to healthy controls, with a difference of 5.5% in the ventral striatum, 26.2% in the pre-commissural dorsal caudate, 29.9% in the post-commissural dorsal putamen, 34.5% in the pre-commissural dorsal putamen, and 60.2% in the post-commissural putamen[232]. Further, in each region of interest, the annual rates of DAT decline were 5.3%, 5.4%, 8.5%, 6.2%, and 7.8%, respectively[232]. This exponential pattern of DAT reduction demonstrates the normal ageing effect in PD[232]. Another study assessed age-related DAT decline in relation to motor function in normal ageing[233]. Although binding potentials of the DAT marker did not vary with age, researchers observed an inverse relationship between the marker for VMAT2 and age[233]. When split into age groups, performance on the motor task positively correlated with age in the younger group and negatively correlated with age in the older group[233]. These results suggest that age-dependent changes in VMAT2 and DAT act independently of one another, and that older individuals experience deficits in motor performance due to a decline in DAT binding[233]. Studies employing positron emission tomography offer further support for the relationship between normal ageing and DAT function, such that the age-dependent decline in DAT availability and binding contributes to impaired motor function in PD.

Ageing-related changes of SNc DAN activity

Results from patch-clamp electrophysiological recordings suggest that DANs within the SNc have similar membrane capacitance and input resistance across age groups[234]. However, ageing leads to slower firing rates, narrower spike widths, variable interspace intervals, and smaller L-type calcium channel currents[234]. Therefore, normal ageing negatively impacts DAN function, impairing voluntary movement among other behavioral processes controlled by DA pathways[234]. Further, vulnerability of DANs within the SNc may be linked to progressive overreliance on L-type calcium channels with normal ageing[235]. This overreliance serves as a chronic stressor on the mitochondrial ATP that drives oxidative phosphorylation, resulting in neurodegeneration[235]. One study employed isradipine treatment to block L-type calcium channels, successfully reversing age-related overreliance on these channels[235]. Therefore, both functional changes in

SNc DANs as well as overreliance on L-type calcium channels occur with normal ageing, resulting in neurodegeneration and motor dysfunction.

Evidence supports the accumulation of various pathological changes in normal postmortem SNc neurons compared to neurons from other brain regions of the same age. Such changes include mitochondria dysfunction, increased protein oxidation, higher levels of astrocytic proliferation, diminished antioxidant function, enhanced oxidative stress, neuromelanin accumulation, inability of neurons to appropriately handle calcium, and increased iron levels[236-238]. The various changes associated with ageing make the nigrostriatal DANs vulnerable to degeneration, and when combined with additional pathologies may ultimately lead to PD[237]. Studies across many animal models have investigated how the multiple processes associated with ageing effect the function and survival of SNc DANs, predisposing them to neurodegeneration. In wildtype mice, ageing alone leads to motor deficits, diminished striatal dopamine levels, fewer DANs, and fragmented mitochondria in DANs[239]. Research with rats have shown that mitochondrial DNA deletions in nigrostriatal DANs increase with age[223]. Studies investigating TH- and neuromelanin-containing DANs in non-human primates show that increasing age is associated with loss of neurons that only contain TH and an increase in neurons that only contain neuromelanin[240]. Age was also associated with loss of dopamine transporter-immunoreactive SN neurons[241]. These changes contribute to functional deficits by reducing striatal dopamine levels in older monkeys[240,241]. Cell vulnerability in response to injury [specifically, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] was also shown to increase with age[240].

The effects of ageing on SNc DANs have also been linked to the diminished expression of specific genes. For example, the importance of *Nurr1* as ageing occurs has been demonstrated in mice[242]. Aged heterozygous *Nurr1*-deficient (*Nurr1*$^{+/-}$) mice showed deficits in rotarod performance and locomotion that were associated with lower levels of striatal dopamine, less nigrostriatal DANs, and less *Nurr1* and DAT expression in the SN compared to wild-type controls. These results suggest an important role of Nurr1 in maintaining nigrostriatal DAN function and survival as ageing occurs. Studies in humans also suggest an important role of Nurr1. The transcription factor Nurr1 is important for establishing and maintaining dopamine phenotypes within the nigrostriatal DANs, and its diminished expression throughout ageing is associated with decreases in TH-positive neurons[243-245]. In PD, SNc DANs with decreased Nurr1 levels were also associated with increases in α-synuclein inclusions[243]. Together, these studies in humans and non-human primates suggests that ageing is associated with a downregulation of genes involved in dopamine transmission[47].

Ageing is also associated with a decrease in the number of DAT-positive SNc neurons[246]. Comparing neurons from young (0-49 years), middle aged (50-69 years), and elderly (70-85 years) human samples show that, by middle age, the number of intensely stained DAT nigrostriatal neurons decreased while the number of lightly stained DAT nigrostriatal neurons increased. Increasing age is also associated with an increase in the number of cell bodies negative for DAT but positive for neuromelanin. Overall, each decade older was associated with a 6.7% decrease in the total number of nigrostriatal DANs.

Ageing-related changes on the function and survival of VTA DANs

Interestingly, motivation has shown to be critical in age-related functional decline of the VTA[247]. In contrast to resting state contexts, VTA and ventral striatum functional coupling was enhanced in adolescence and decreased in adulthood in a motivational context, suggesting a distinguishing ageing marker[247]. Moreover, a decline of DAN function has been behaviorally associated with deficits in learning[248]. In terms of electrical physiology, the frequency of burst events of VTA DANs did not change

with age, but bursts were longer in adolescents than in adults, potentially because GABA tone increases as rats reach adulthood. The firing rate increasing in adolescence is consistent with it being a more vulnerable time for developing drug addiction.

There are sex differences in functional connectivity of the VTA. For instance, men have a stronger VTA/SNc connectivity to the left posterior orbital gyrus than woman according to a study that measured resting state blood oxygenation level dependent signals[249]. Moreover, only men showed age-related functional VTA changes to cortical and cerebellar regions, implying that ageing differentially affects not only sexes but also distinct cerebral projections[249]. In a study on human post-mortem brain samples, there was no statistically significant loss of VTA DANs as a function of age, suggesting that the neurodegeneration implicated in ageing and PD is a result of but not the initiating cause of neuronal death[250]. However, more studies will be required to critically evaluate the function and survival of different VTA DAN subtypes during the normal ageing process.

CONCLUSION

In the past decades, tremendous progress has been made in understanding the neurological and genetic causes of PD-related motor and non-motor impairments[2], as well as how the different facets of the ageing process contribute to the progressive dysfunction and loss of DANs. However, due to a lack of distinctive molecular markers, the SNc or VTA DAN subpopulations were often studied as a homogenous unit, although many of these neuron subtypes display distinct connectivity, functionality, and susceptibility to ageing and PD. With the advance of single cell RNA sequencing technology, increasing numbers of molecularly defined midbrain DAN subtypes have been identified[35]. By employing intersectional genetic approaches, recent studies managed to genetically distinguish different midbrain DAN subpopulations. Various live imaging techniques with different genetically encoded sensors make it possible to directly correlate the neuron activity with behaviors and longitudinally monitor the neuron activity during ageing. The development of CRISPR/Cas9 gene editing, optogenetics, and chemogenetics procedures allow researchers to establish a causal relationship between neural activity and behavioral performance through genetically and functionally manipulating the neural activity. The knowledge gained from these ongoing studies may explain how different subtypes of DANs contribute to different aspects of behavioral phenotypes and provide new mechanistic insights into novel procedures for reconfiguring PD-induced behavioral abnormalities.

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Authors' contributions

Conceived the theme of this review article, wrote the abstract, introduction, and conclusion sections, and made the figures: Cai H
Contributed to the molecular genetics section: Sun L
Contributed to the SNc, PD and ageing sections: Carmichael K
Contributed to the VTA section: Lopez E
Contributed to the PD and ageing section: Sullivan B

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Ageing and Neurodegenerative Diseases

Review

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Naive BM-derived stem cells (Neuro-Cells) may modify acute and chronic neurodegenerative disorders by modulating macrophage behaviors

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Abstract

In acute traumatic or hypoxic brain and spinal cord lesions, as well as in chronic idiopathic neurodegenerative disorders induced by a genetic/environmental/idiopathic protein misfolding with aggregation, emerging evidence indicates that primary necrosis, as induced by the underlying event, initiates a secondary inflammatory process. In this secondary process, responsible for significant neurological deterioration, a microglia type M1/M2 misbalance plays a major role. Indeed, both acute and chronic neurodegenerative disorders share a common pathway: a M1/M2 misbalance-induced hyperinflammatory process with a lack of response to conventional anti-inflammatory interventions. In recent literature, however, both in preclinical and clinical neurodegenerative conditions, these processes were suggested to be sensitive for interventions with stem cells. Intrathecal interventions with a fresh, not-manipulated (naïve) bone marrow-derived stem cell preparation, after positive selection of pro-inflammatory substances (Neuro-Cells), were found to prevent/reduce secondary necrosis-induced pro-inflammatory and pro-apoptotic processes in both immune-compromised and otherwise healthy experimental animal models. Therefore,

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it seems justified to further encourage clinical trials applying autologous BM-derived naïve stem cells in patients suffering from those debilitating neurodegenerative conditions.

Keywords: Naïve bone marrow-derived stem cells, Neuro-cells, M1/M2 paradigm, cytokine release syndrome, neurodegenerative disorders

INTRODUCTION

In any acute or chronic, systemic or compartmental insults, interferons produced by lesioned cells are responsible for a range of signaling events leading to an inflammatory process, eventually ending with apoptosis and/or necrosis. Chemokines will activate immune cells such as macrophages and microglia to travel to the site of the insult. Exposed to inflammatory stimuli, these cells will initiate the secretion of cytokines.

Adequate resolution of the inflammatory process with phagocytosis of cell debris, cell survival and tissue repair, will be reached when the temporal dynamics of these cytokines show an early innate immune response with a release of pro-inflammatory tumor necrosis factor (TNF)-α, followed by a release of interferon (IFN)-γ, and then mainly interleukin (IL)-1β, IL-6, and IL-12. Normally, an adequate number of anti-inflammatory cytokines such as IL-4 and IL-10 will be subsequently released in response to the prior pro-inflammatory cytokines. However, an inadequate counter-balancing level of anti-inflammatory cytokines, or an overzealous/prolonged secretion of pro-inflammatory cytokines might cause a vicious, hyperinflammatory cycle [Figure 1]. Increasing inappropriate cytokine release-related morbidity includes multi-organ failure, neurotoxicity, and death[1-2].

Indeed, a systemic or compartmental disbalance between pro- and anti-inflammatory cytokines (i.e., a disturbed microglia type M1/M2 balance) may result in hyperinflammatory conditions and/or cytokine release syndromes (CRS). These conditions might be best formulated as an infectious or otherwise-induced production of circulating cytokines beyond a normal response, leading to inflammatory signs with fever, severe fatigue, nausea, and in some cases even secondary organ dysfunction or multi-organ failure[3]. Infectious insults include sepsis, viremia, herpes, Ebola, malaria, Dengue, Lassa, and coronavirus-induced severe acute respiratory syndrome or Middle East respiratory syndrome. Sterile conditions, such as monogenic disorders, autoimmune diseases, organ transplantation, immunotherapies like monoclonal antibodies or chimere antigen receptor-T cells for cancer, as well as burns, ischemia, and trauma, may also initiate inappropriate cytokine secretion[4-6]. The insults may be acute, subacute, or chronic. In chronic neurodegenerative disorders, chronic misfolding of proteins with subsequent divergent accumulation and aggregates formation as well as ongoing cell necrosis can be related to a disturbed M1/M2 paradigm with an elevation in the M1 pro-inflammatory phenotype by the continuous exposure to pathogen-associated molecular patterns (PAMPs) and/or endogenous damage-associated molecular patterns (DAMPs). A similar shift towards M1 polarization might also be seen in rheumatoid arthritis arthritis[7].

CRS manifest with fever and general malaise, but as soon as endothelial cells become involved it may also come with coagulopathy, capillary leaks, and disruption of membranes. Also membranes surrounding immune-privileged compartments such as the blood-brain-barrier might be affected and lose their relative impermeability, thus enabling immune cells to freely pass those membranes[8]. The massive intracerebral influx of macrophages due to this increased permeability of the blood-brain-barrier explains the progressive exacerbation in chronic progressive neurodegenerative disorders. If untreated, most patients will suffer a diffuse intravascular coagulation and/or a pro-thrombotic coagulopathy with thrombocytopenia, leading to

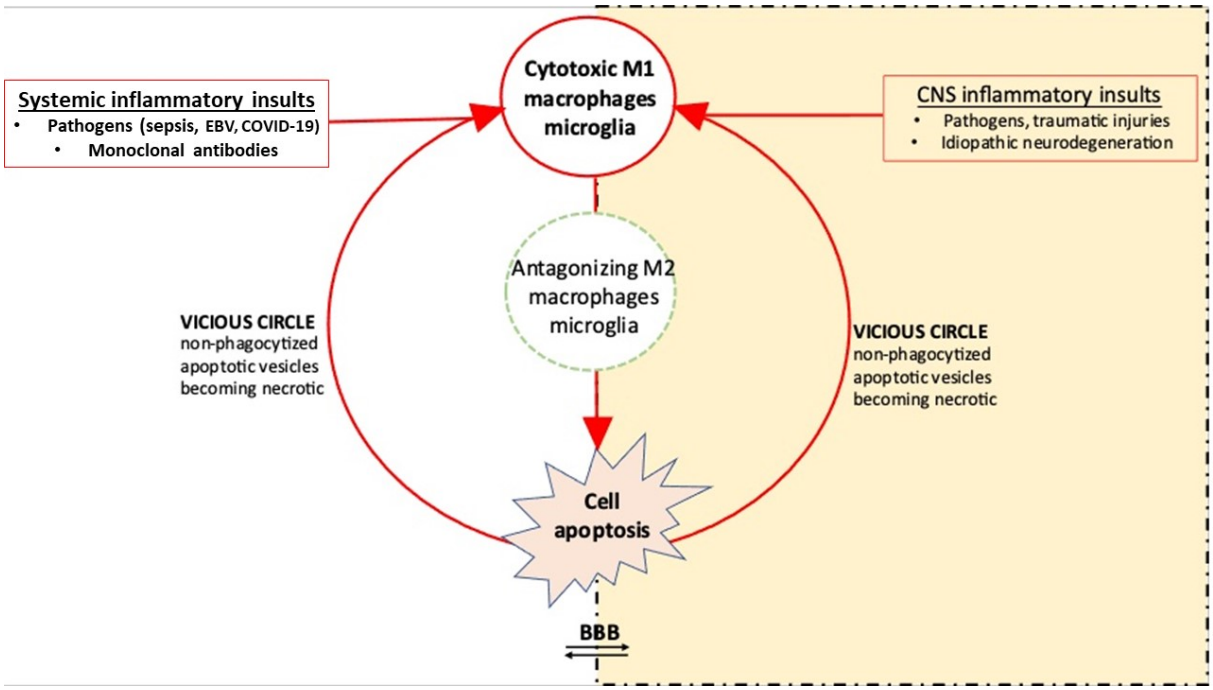

Figure 1. The origin and propagation (vicious cycle) of hyperinflammation. In case of hyperinflammation, inappropriate (increased) levels of pro-inflammatory cytokines or (decreased) levels of anti-inflammatory cytokines (M1/M2 paradigm) initiate a vicious circle as non-phagocytized apoptotic vesicles become necrotic and continue the activation of resting macrophages/microglia into M1 macrophages/microglia. Thus, ongoing signaling pathways prolong the cytokine cascade with activation of other immune cell types which promote cell proliferation, boost the pro-inflammatory cytokine release (mainly IL-6), and thus promotes the propagation of tissue damage. In the figure, the blood-brain-barrier (BBB) is shown with the systemic compartment (blood) on the left and the CNS compartment at the right side. In case of hyperinflammation, BBB permeability is increased.

hypotension, multi-organ failure and/or acute hypoxemia[1].

In the absence of external stimuli, macrophages and microglia are normally in a resting state (M0 macrophages/microglia). Due to polarization, macrophages adopt different functional programs in response to microenvironmental signals [Figure 2]. Pending their micro-environment and the presence of polarizing cytokines, they may be classically activated into M1 phenotypes, as well as into alternatively activated into M2 phenotypes. In the M1 state, macrophages/microglia secrete pro-inflammatory responses, enhancing nitric oxide synthase. In the M2 state, in addition to stimulating responses for repair and recruitment (from M2a and M2b phenotypes, respectively) they also may secrete anti-inflammatory phagocytic responses (M2c phenotypes)[9]. Indeed, macrophages also play an important role in the embryonic development, removal of cellular debris, and tissue repair. The polarization of mononuclear macrophages into M1 or M2 macrophages is a simplified conceptual framework to describe their plasticity[10]. Originally, macrophages were thought to be activated by IFN-γ alone or in concert with microbial stimuli (e.g., lipopolysaccharide) or cytokines (e.g., granulocyte-macrophage colony-stimulating factor) (so called classically activated macrophages). Subsequently, macrophage colony stimulating factor, TNF-β and the interleukins IL-4 and IL-10, rather than inhibiting this classical activation, were found to induce an alternative (M2) form of macrophage activation [Figure 2]. In response to certain endogenous and exogenous conditions, macrophages may even reverse classical or alternative polarization.

Although different patterns of macrophage responses cannot always be accurately described along the M1/M2 axis (in some reactive microglial populations, the canonical gene products of both "polarized" states

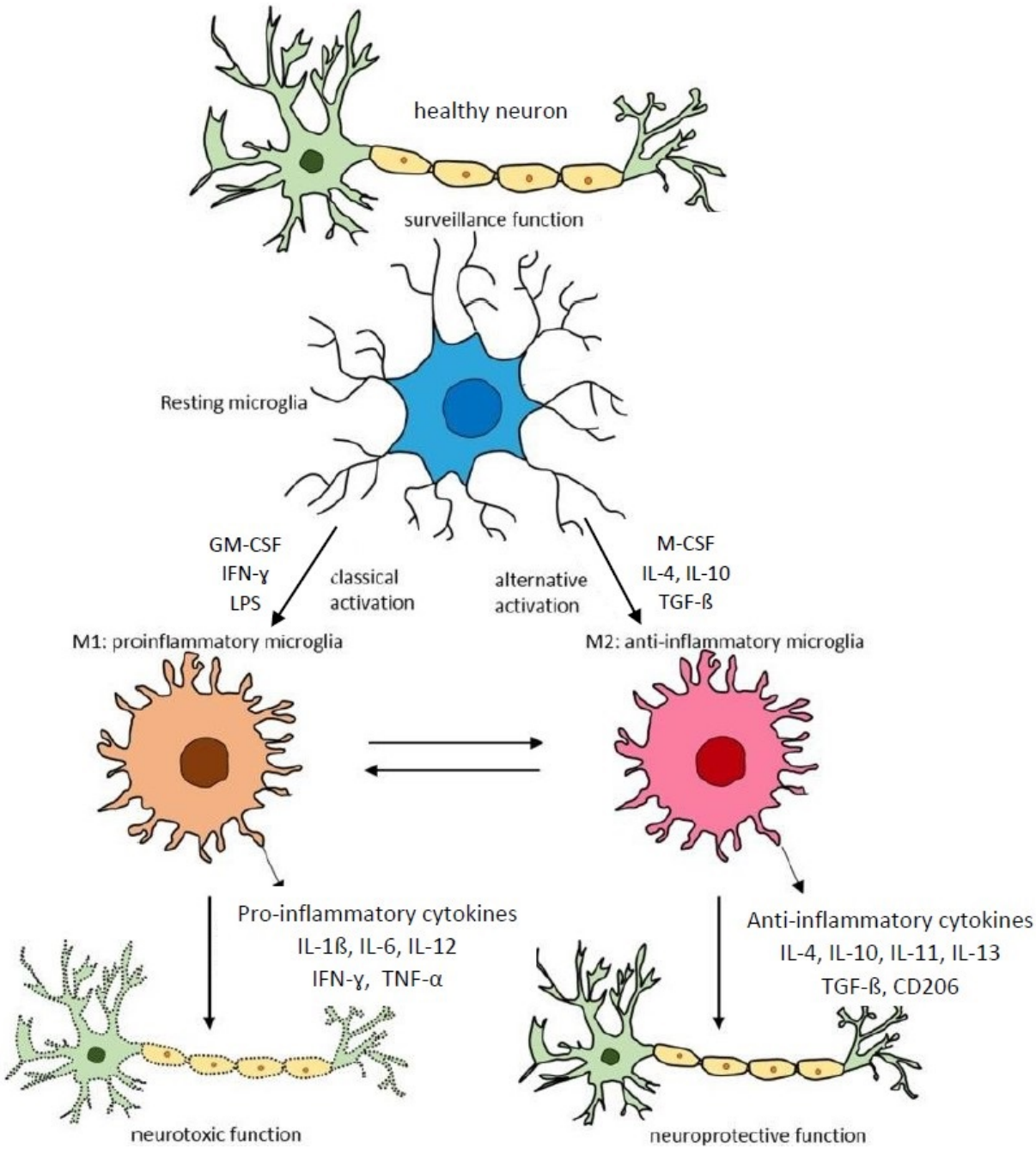

Figure 2. The M1/M2 paradigm in the central nervous system. Transcriptional regulators of M1 and M2 activation of microglia and mechanisms of their stimulation/inhibition. Resting microglia are stimulated by interferon (IFN)-γ, lipopolysaccharide (LPS), and/or granulocyte-macrophage colony-stimulating factor (GM-CSF) to classical activation into M1 microglia, and by macrophage colony-stimulating factor (M-CSF), IL-4, IL-10, and transforming growth factor (TGF)-β for alternative activation into M2 microglia, producing pro-inflammatory and anti-inflammatory cytokines, respectively. In a well-balanced M1/M2 condition, there will be an adequate resolution of the inflammatory process. TNF-α: Tumour necrosis factor α; CD206: macrophage mannose receptor type 1 (adapted from Subramaniam *et al*.[9]).

are co-expressed)[11], the M1/M2 axis simply reflects the most phenotypically polar differentiation states of macrophages and, therefore, is often implied in research. In *in vivo* studies, the M1/M2 dichotomy may possibly be replaced with the terms pro-inflammatory/pro-regenerative[12].

In the acute phase of an insult, M1 macrophages phagocytose the debris and promote the flow of other immune cells by expressing pro-inflammatory cytokines, mainly IL-1β, IL-6, IL-12, TNF-α, and IFN-γ [Figure 2].

After the acute phase with the onset of classical pro-inflammatory activation of the resting homeostatic M0 macrophages/microglia into M1 phenotypes, normally within 3-7 days later, the transition to regeneration is reflected by increasing numbers of alternatively activated (M2) macrophages, dampening the pro-inflammatory M1 cells-induced immune responses, and promoting regeneration and angiogenesis by expressing anti-inflammatory cytokines such as tumor growth factor (TGF)-β and the interleukins IL-4, IL-11, IL-13, and especially IL-10 [Figure 2].

In the recent past, more attention is given to the role of neuro-inflammation as a common final pathway in neurodegenerative disorders. Indeed, neuro-inflammation (i.e., gliosis and inflammatory reactions) has been described as a prominent sign in Alzheimer's disease[10,13], Parkinson's disease[14], Huntington disease[15], amyotrophic lateral sclerosis[16], prion disease[17], and multiple sclerosis[18,19]. In these disorders, chronic protein misfolding maintains a disturbed M1/M2 paradigm by the continuous exposure to PAMPs and/or DAMPs.

INAPPROPRIATE CYTOKINE RELEASE SYNDROMES

Macrophages and microglia are the sentries of the innate immune system in injury and infection; they are thought to play a major role in the tissue and organ homeostasis, as well as in autoimmune diseases, atherosclerosis, and cancer.

The timely switching of macrophage polarization from M1 to M2 plays a major role in the outcome of the inflammatory reaction (regeneration or fibrosis). Adequate immunosuppression and neuron protection is pending from a normal M1/M2 paradigm[10]. In case of a disturbed paradigm, a necrotic cell-induced hyper-inflammatory condition may result, due to a vicious circle with an ongoing classical activation of the microglia, a condition with a great deal of collateral damage[20-22] [Figures 1 and 3].

Both in systemic and CNS (central nervous system) compartmental inappropriate CRS, the blood-brain-barrier permeability normally preventing for the infiltration of blood-borne monocytes/macrophages may be compromised, allowing the passage of chemokines, immune cells, and cytokines[8,23-28]. Thus, systemic hyper-inflammation may manifest with encephalopathic manifestations and a permanent deterioration in pre-existing neurodegenerative disorders[2,29,30].

In recent years, in several neurodegenerative diseases, the M1/M2 paradigm of microglial activation was extensively studied to uncover the mechanisms of immunopathogenesis. Molecular and clinical evidence from positron emission tomography imaging and post-mortem analysis suggested an increase of microglial activation and inflammatory mediators during the pathogenesis in these disorders[10]. Predicting the presence and severity of CRS has also been a challenge because this syndrome starts in the target tissue(s), only coming to attention when damage has occurred[1,8]. CRS is a dynamic process and body fluid cytokine levels may not adequately reflect the actual underlying physiological processes[31]. Nevertheless, peripheral blood biomarkers of CRS, even reflecting the remaining situation after cell redistribution to tissues or cell death, are used for diagnosis and to guide therapy[32].

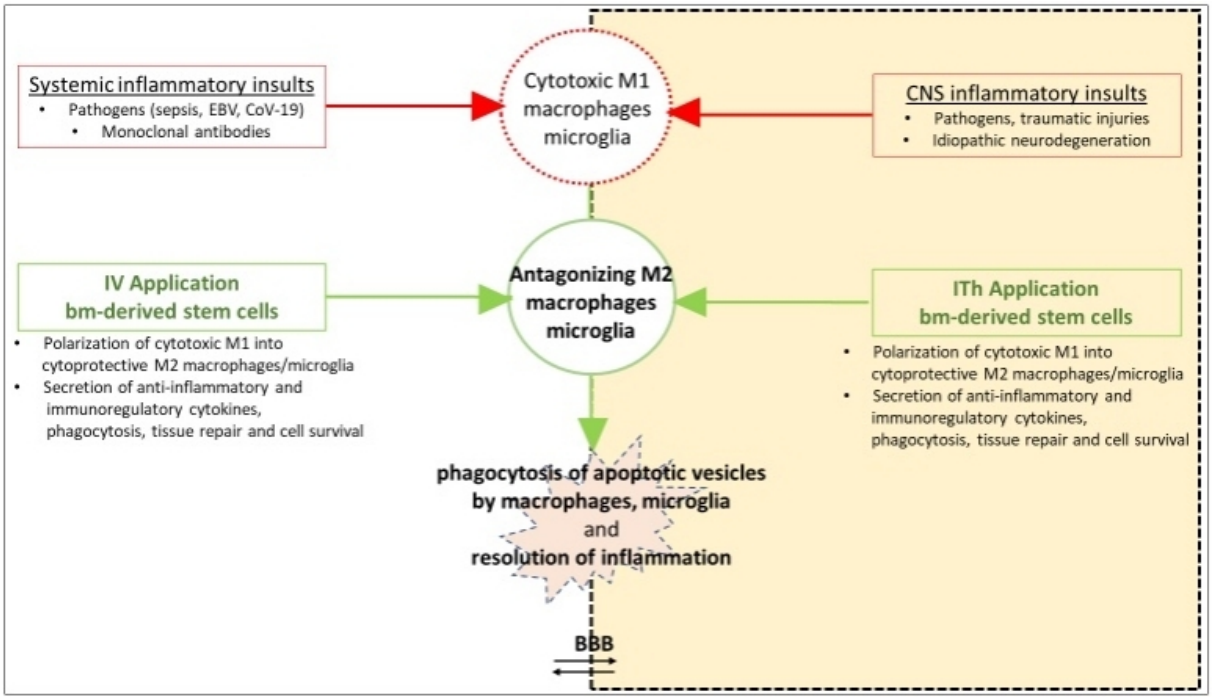

Figure 3. The breaking of the vicious cycle in hyperinflammatory conditions by bone marrow-derived naive stem cells. Necrosis in the target tissues initiate an M1 macrophage/microglia-induced cytokine cascade and activation of other cell types resulting in cell proliferation, further pro-inflammatory cytokine release (mainly IL-6), and propagation of tissue damage. In case of an imbalanced release of pro- and anti-inflammatory cytokines, phagocytosis of the apoptotic vesicles is blocked, and a vicious cycle will follow by the non-phagocytized vesicles, becoming necrotic. Intravenous (IV) and/or intrathecal (ITh) transplantation of stem cells then may restore the imbalanced cytokine levels and break the vicious cycle by polarization of cytotoxic M1 into antagonizing M2 immune cells on the one, and inhibition of the up regulation of the protein expression of inflammatory markers (GSK-3β) on the other hand.

INAPPROPRIATE CYTOKINE SECRETION IN NEURODEGENERATIVE DISORDERS

Neurodegenerative disorders are hereditary and/or sporadic, acute and/or chronic conditions, characterized by nerve cell degeneration and/or necrosis due to atrophy of the nervous system, interfering with normal mental and motor functioning.

Alzheimer's disease

Alzheimer's disease (AD) is an age-related multifactorial genetic/environmental neurodegenerative disorder resulting in a progressive impairment in memory, judgement, decision-making, and orientation. In this disorder, intracellular, misfolded tau protein-containing tangles underlie the neurofibrillary degeneration. Microglial macrophages react to the amyloid β peptide by releasing pro-inflammatory factors, promoting their own phagocytic activity[33]. In the immediate vicinity of the characteristic amyloid peptide deposits and neurofibrillary tangles, primarily pro-inflammatory (IL-1ß, IL-6, IL-12, and TNFα) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) were found to play a major role in the phagocytic clearance of apoptotic neurons, indicating that inflammation, indeed, is a key pathological hallmark of AD. In Alzheimer's disease, tau phosphorylation is thought to be responsible for the M1-activated microglia-induced neurotoxicity ease[33-36].

Parkinson's disease

Parkinson's disease (PD) is also an age-related genetic/environmental disorder. It is clinically characterized by hypokinesia, bradykinesia, rigidity, and tremor as well as numerous autonomic and mental symptoms, evidencing a multisystem α-synucleinopathic neurodegenerative process. The abundant synuclein

characteristically aggregates in Lewy bodies. Both direct and indirect microglial activation are initiated by aggregated α-synuclein. Numerous studies have shown that α-synuclein, probably by its dysregulation of the JAK/STAT pathways in myeloid cells[37], directly activates microglia into the M1 phenotype, with the activation of NADPH oxidase, and increasing production of reactive oxygen species and pro-inflammatory cytokines[38]. In PD patients, increased levels of immune cells and proteins such as adhesion molecules, chemokines, cytokines, and decreased levels of neurotrophins in brain, spinal fluid, and serum, such as brain-derived neurotrophic factor and nerve growth factor, evidenced chronic cytotoxic classical microglial activation with apoptotic cell death[39-43].

Huntington's disease

Huntington's disease (HD) is a progressive autosomal dominant monogenic disease, displaying a selective striatal and cortical neuronal loss, manifesting with a progressive motor dysfunction, cognitive decline, and psychiatric disorders. HD is caused by CAG trinucleotide repeat expansion in the gene encoding for huntingtin protein on chromosome 4p16[44]. In HD, proteomic plasma profiling demonstrated that increasing cytokine levels antedate the onset of neurological symptoms. Both in HD patients and experimental animal models, CNS microglial activation was found to result in an increased production of inflammatory mediators, and TNF-α and IL-6 mRNA levels were found markedly increased[45,46].

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a mainly sporadic (about 5%-20% familial) multifactorial disease caused by motoneuron degeneration in the spinal cord, brain stem, and primary motor cortex, with cytoplasmic inclusions containing aggregated/ubiquitinated proteins as well as RNAs. In this disease, again, glial activation leads to changes in the expression of a wide range of genes related to the production of soluble molecules, such as cytokines, chemokines, DAMPs, and reactive nitrogen and oxygen species, giving rise to profound modifications in their interactions with neurons[47].

Multiple sclerosis

Multiple sclerosis (MS) is the most common genetic/environmental chronic inflammatory disorder of the CNS, which may manifest as a relapsing-remitting or a secondary progressive disorder[19]. The infiltration of increased autoreactive myelin-specific CD4 and CD8 T helper cells into the CNS represents the crucial event in the inflammatory processes with the formation of focal inflammatory demyelinated lesions (plaques) via the secretion of M1-produced IFN-γ and IFN-γ-promoted TNFα[48-50].

Spinocerebral injuries (SCI) display evidence indicating that immediately after the trauma, macrophages accumulate within the epicenter of the lesion and may initiate necrosis-induced secondary M1 promoted inflammatory mechanisms, overwhelming a comparatively smaller and transient M2 macrophage response, leading to cavities and scar tissue. In time, the acutely increased levels of TNF-α, IL-1β, IFN-γ, and other pro-inflammatory M1-produced cytokines, chemokines, and proteases will gradually decrease, and increasing numbers of anti-inflammatory M2 cytokines will restore the initial M1/M2 balance[51]. Normally, *in vitro*, myelin phagocytosis comes with a facilitation of M2 polarization; macrophages in a damaged spinal cord are strongly inclined towards M1 polarization, which interferes with the neural tissue recovery.

STEM CELLS

Stem cells are essential for the development, assembling, and repairing of bodily structures. Without these cells one cannot survive. Recently, these cells emerged as a promising tool for the modulation of the immune system. They are undifferentiated cells that not only may proliferate, but also are able to differentiate into all kinds of target cells. Stem cells can be harvested out of adipose tissue, bone marrow, olfactory mucosa, umbilical cord blood, and embryonic tissue, as well as out of special niches in organs, all

varying in their regenerative capacity and potency. Their specific differences in biological properties might be an important consideration for their selection in regenerative medicine[52]. Applying autologous stem cells is preferred over allogeneic preparations, as these come with a risk of immunological incompatibility. In regenerative medicine, mostly bone marrow-derived stem cells are applied. Another source of stem cells is supplied by reprogramming adult somatic cells back into pluripotent stem cells: induced pluripotent stem cells. To reach quantitative numbers of stem cells, culturing these cells might help, though this procedure may come with changes in the telomers. Compared to small molecules such as neurotransmitters, and biologics such as antibodies, growth factors, and/or cytokines, stem cells act fundamentally different. However, the exact mechanisms of action of stem cells remain to be elucidated.

The nervous system, unlike many other tissues, has a limited capacity for self-repair; mature nerve cells lack the ability to regenerate, and only neuronal-resident stem cells have the potency to generate new functional neurons in response to lesions. Their limited availability, though, makes them unfit to cure devastating neurodegenerative diseases. Circumvention of this problem through intracerebral neuronal-resident stem cells grafts, on the other hand, raises serious concerns since the pathological phenotype of the diseased endogenous cells may affect the graft tissue. Neuronal-resident stem cells are already predestinated for neuronal renewal-committed operations, whereas naïve stem cells (with an excellent self-renewal capacity with sustained multipotency) are still multi-potent and also exert, for instance, immune-suppressive effects as a dedicated reaction to environmental vesicles or cytokines from degenerating, malfunctioning cells[53].

As said before, originally, the mode of action of bone marrow-derived stem cells was thought to be related to cellular integration by leveraging the plasticity of the stromal/stem and progenitor cells for the replacement of lost cells. Later, the mechanism was also considered to relate indirectly via cellular interactions. As stem cells hardly pass the intact brain barriers, eventual immunosuppressive paracrine and endocrine effects of stem cells in neurodegenerative conditions are rather reached through cell-to-cell interactions by communicators, signaling proteins such as extracellular vesicles, cytokines, growth factors, and/or mitochondrial transfers. Stem cell extracellular vesicles, indeed, were found to exert immune-suppressive effects as a dedicated reaction to environmental vesicles or cytokines from degenerating, malfunctioning cells, thus coordinating their operations with their immediate environment[54-56]. They might be seen as decision making cells. For example, a high concentration of interferon-γ can activate the naïve stem cells to inhibit the innate immune responses, whereas a low concentration will result in the reversed effect.

Autologous stem cell transplants were found to modulate the immune system in both acute[57-59], and chronic[60,61] preclinical and clinical neurodegenerative conditions.

To assure that those stem cells can adapt to local circumstances, it is crucial not to change the multi-potent characteristics of these cells before the cells are re-implanted in the patient. Stem cells have a variety of receptors on their surface, which can be activated by specific antibodies, each changing the polarization of the cell and thus its naïve status[62]. In order to apply naïve stem cells into the environment where neuroinflammation and degeneration are ongoing, in our experiments, fresh human bone marrow-derived stem cells specimen with negatively selected stem cells were manufactured after positive depletion of erythrocytes, monocytes, and lymphocytes, and reduced in volume for intrathecal application (Neuro-Cells: patent WO2015/059300A1). Indeed, intravenous application will end up with most stem cells stuck in lung and liver, and the number of engrafted stem cells reaching the central nervous system will be minimal. Neuro-Cells, intrathecally applied, appeared to be a safe and effective treatment in preclinical models of neurodegeneration as well as in patients. The number of fresh bone marrow-derived stem cells (100 mL

bone marrow contains about 10^8 CD34+ cells) is limited, though, and their half-life is about 72 h. In cases with reduced plasticity of stem cells (e.g., diabetes, renal failure, aging, and severe amyotrophic lateral sclerosis), one may thus consider applying allogenic cells.

As the effects of the stem cells are thought to be reached by cell-to-cell reactions, not the dose but rather the timing is key, as an effective treatment window in acute neurodegenerative processes lies between 24 to 72 h after the initial CNS insult. Similarly, in chronic conditions with ongoing necrosis of neural cells, the best strategy appears to be starting treatment as early as possible, assuming that dead neurons cannot be replaced with this therapy. Here, the key is the slowing down of the ongoing and self-reinforcing disease process by applying the stem cells as early as possible.

STEM CELLS IN THE TREATMENT OF NEURODEGENERATIVE DISORDERS

Regarding systemic CRS, apart from specific vaccines and maybe the anti-viral remdesivir and/or dexamethasone for treatment of some virus-induced syndromes, there are no convincing disease-modifying interventions for those conditions, and symptomatic treatments are still enigmatic. Also, in the treatment of compartmental release syndromes such as in neurodegenerative disorders, due to ambiguous effects and/or serious adverse events, interventions with anti-inflammatory (non)-steroidal anti-inflammatory drugs [(N)SAIDs] were not very successful[63].

As most of acute and chronic, systemic and compartmental CRS, irrespective of their cause, share a common pathophysiological pathway [Figure 1], it seems justified to treat those conditions, in the same way, regardless the phase of the immunological response[2]. Here, adequate understanding of the role of chemokines and cytokines is important for better understanding these syndromes, as well as for diagnostic purposes and the development of therapeutic options. In modern biomedicine, as of now, regulation of cell homeostasis by modulating macrophage behavior in different pathological conditions is key. The M1/M2 paradigm allows the reassessment of the course of typical pathological processes in terms of a misbalanced M1 and M2 macrophage polarization. Here, increasing the relatively low level of M2 macrophage/microglia phenotypes, for instance, might further stimulate regeneration, angiogenesis, and extracellular matrix remodeling. So, in CRS, restoring the M1/M2 phenotype balance might thus lead to restoration of homeostasis and improved clinical symptoms[64]. As pro-inflammatory macrophages are abnormally overrepresented in acute and chronic neurodegenerative disorders, in the next future molecular interventions affecting the M2 subpopulation, therefore, may offer a potential efficient therapeutic approach to suppress or boost the expression of certain genes in these conditions in order to obtain stably polarized M1 or M2 species[49]. The eventual incorporation of cytokines into therapeutic regimens, though, has significant challenges. In addition to low response rates when administered as recombinant proteins and short half-life limiting exposure and efficacy, cytokines can also activate counterregulatory pathways (i.e., immune-potentiating cytokines might initiate immune suppression), thus limiting their potential efficacy[65].

Recent approaches with stem cell implants yielded promising results in patients suffering acute[57,66] and chronic[60,67,69] neurodegenerative disorders. Our own preclinical studies in animal models of acute traumatic spinal cord injury[59,69] and chronic neurodegenerative processes such as amyotrophic lateral sclerosis and frontotemporal lobe degeneration[58,61] were fully in line with these findings. In these experiments, intrathecal application of Neuro-Cells in the various experimental animal models were found to break the hyper-inflammatory process by restoring the normal M1/M2 paradigm [Figure 3].

Those stem cells, but not (N)SAIDs, significantly improved the functional outcome and reduced signs and symptoms of inflammation in these animal models, compared to those treated with placebo, and were free

of adverse events. Intrathecal application of Neuro-Cells in SCI-rats within 24 h after the lesioning, induced depolarization of M1 into M2 reactivated macrophages/microglia, thus preventing for the secondary inflammation-induced elevations in serum IL-1β, TNF-α, and IL-6 levels as well as for the elevation of glycogen synthase kinase (GSK)-3β and ionized calcium-binding adaptor molecule (Iba)-1 protein levels in the spinal cord. Those stem cells were found to reduce the SCI-induced downstream IL-6 signaling pathways with cytokine-driven hyperimmune reaction[59,69]. Compared to vehicle-treated animals, immunohistochemical analysis 4 days after the intervention, but not 8 weeks later, displayed a significant increase of $CD68^+$ microglia ($P < 0.01$) and decrease of $GFAP^+$ expression of astrogliosis in the lesion ($P < 0.05$), as well a reduced apoptosis with a significant decrease in cleaved caspase-3^+ cells, compared to vehicle-treated SCI-rats. Eight weeks after these interventions, though, histological studies of the lesioned tissue in the Neuro-Cells and vehicle-treated SCI-rats did not establish any significant difference any more in the expression of microglia, astrocytes, and apoptosis. Compared to the baseline in vehicle-treated animals (set to 100%), proteomics in the Neuro-Cells-treated rats at that time still showed significant changes in the downregulation of pro-inflammatory proteins and the upregulation of the proteins involved in axonal and cellular regeneration [Figure 4]. An interesting finding was also the significant lower expression of Iba-1 in the spinal lesion of the Neuro-Cells-treated SCI-rats, compared to these animals treated with vehicle and/or intraperitoneal methylprednisolone, 10 weeks after these interventions [Figure 4].

Conforming to previous studies with stem cell implantations[70-72], intrathecal Neuro-Cells implants, but not interventions with riluzole and/or celecoxib in 10-week old asymptomatic FUS(1-358) and SOD1(G93A) mutant ALS-like mice were found to significantly delay motor dysfunction, as well as muscle atrophy and the loss of spinal lumbar motor neuron as seen in transgenic mice[61]. Interventions with Neuro-Cells in 12-week old asymptomatic FUS(1-358) frontotemporal lobe degeneration-like mice significantly delayed signs of depression and anxiety, cognitive deficits, and abnormal social behavior compared to FUS-tg placebo-treated animals. Neuro-Cells did normalize prefrontal and hippocampal protein expression of IL-1β, and of hippocampal Iba-1 and GSK-3β. In these transgenic mice, interventions with riluzole and celecoxib did bring the same beneficial effects, though way less pronounced[73].

SUMMARY AND CONCLUSION

Both in acute traumatic or hypoxic neurodegenerative lesions, as in chronic protein misfolding-induced neurodegenerative disorders, emerging evidence indicates that primary necrosis as induced by the underlying event initiates a secondary inflammatory process by a M1/M2 misbalance. This secondary process is responsible for a significant increase in the ultimate neurological deficit. These neurodegenerative diseases share a final common pathway, that is a M1/M2 misbalance-induced autoreactive response that targets against components of the nervous tissue.

Here, an up-regulated protein expression of inflammatory markers, GSK-3, regulating several signaling pathways including pro-inflammatory cytokine and interleukin production in the innate immune response, and Iba-1, a marker of microglia activation, reflects this degenerative process. These conditions also share an unmet need for disease-modifying interventions.

In this article, we presented data of preclinical studies after the effects of intrathecal implants of a human bone marrow preparation with truly naïve, negatively selected stem cells (Neuro-Cells) in rat models of spinal cord injuries, and in SOD-1 and FUS-transgenic amyotrophic lateral sclerosis-like and frontotemporal lobe degeneration-like mice. Neuro-Cells implants induced disease-modifying effects with changes in markers for M1/M2 macrophages/microglia, where (N)SAIDs failed. Indeed, both in immune-compromised and otherwise healthy experimental SCI-lesioned rats and in ALS- and frontotemporal lobe

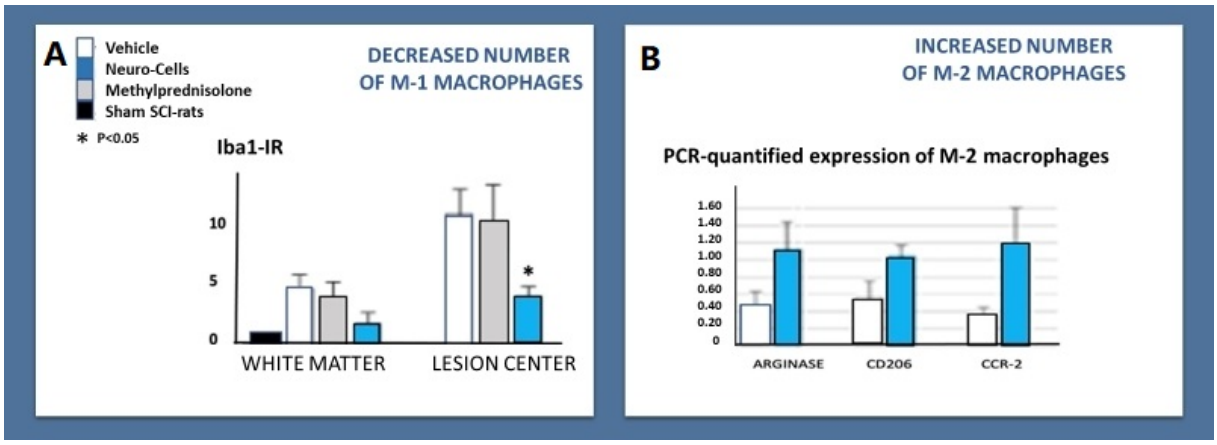

Figure 4. Changes in the M1/M2 paradigm 8-10 weeks after an intervention with bone marrow-derived stem cells (Neuro-Cells), methylprednisolone and vehicle in the acute phase of a spinal cord injury in rats. (A) The Western blot Iba1-IR quantified polarization from M1 to M2 microglia in the spinal cord white matter within the area close to and into the lesion center. Ten weeks after the intrathecal intervention with Neuro-cells, the Iba1 expression was significantly lower ($P < 0.05$) compared to the treatment with intrathecal vehicle and/or intra-peritoneal methylprednisolone 150 mg/kg (data normalized to Iba1-IR in the spinal white matter of intact healthy (sham) rats; statistical difference not indicated). Bars show means and SEM, n = 5-6 rats/group (Romero-Ramírez *et al.*[61], 2020, with permission of the authors). (B) Display of the increased levels of the typical M2-synthesized arginase-1 (inhibiting NO production), the M2 cell surface marker CD206 and the chemokine receptor CCR-2, polarizing macrophages toward an M2 phenotype, PCR-quantified polarization from M-1 to M-2 microglia (adapted from Wolters *et al.*[65] and de Munter *et al.*[71] with permission of the authors), 8 weeks after the acute intrathecal intervention of Neuro-cells in acute balloon compression-induced spinal cord injured rats, compared to SCI-rats, treated at the same time with only the vehicle. Due to the low numbers of experimental animals, significances were not reached.

degeneration-like transgenic mice, intrathecal Neuro-Cells implants prevented for outrageous secondary inflammatory and apoptotic effects as evidenced by elevated GSK-3β and Iba-1 protein levels in the CNS. Therefore, it seems justified to further encourage clinical trials, applying bone marrow-derived naïve stem cells in patients suffering debilitating neurodegenerative diseases.

DECLARATIONS

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Authors' contributions

Participate in a close-knit international research team that performed the preclinical research discussed in this review article dealing with the effects of mesenchymal stem cells in neurodegenerative disorders: Wolters EC, Strekalova T, de Munter JPJM, Kramer BW

Availability of data and materials

Not applicable.

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Conflicts of interest

In addition to a staff position at the University of Maastricht, The Netherlands (Department of Neuroscience and Mental Health), Johannes de Munter is CEO of Neuroplast BV (the manufacturer of Neuro-Cells). Otherwise, there are no conflicts of interest whatsoever.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Mild cognitive impairment *vs*. mild cognitive dysfunctions: validation with a nomothetic network approach

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Abstract

Aim: No studies have examined whether interactions between the apolipoprotein E4 (ApoE4) allele and peripheral biomarkers, hypertension, and type 2 diabetes mellitus (T2DM) may impact the neurocognitive, behavioral, and social dysfunctions in amnestic mild cognitive impairment (aMCI) and Alzheimer's disease (AD). We aimed to clinically define and biologically validate a subgroup of aMCI subjects who take up an intermediate position between controls and AD patients.

Methods: In 61 healthy controls, 60 subjects with aMCI, and 60 AD patients, we measured the features of aMCI/AD using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD). A composite BIORISK score was computed using the ApoE4 allele, serum folate, albumin, white blood cells, fasting blood glucose, atherogenic index of plasma, T2DM, and hypertension.

Results: Clustering and nearest neighbor analyses were unable to validate the aMCI subgroup. We constructed two z unit-based composite scores, the first indicating overall burden of cognitive, social, and behavioral deterioration (OBD) and the second reflecting the interactions among ApoE4, all other biomarkers, hypertension, and T2DM (BIORISK). We found that 40.2% of the variance in the OBD score was explained by BIORISK, ApoE4,

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age, and education. The OBD index was used to construct three subgroups (normal, medium, and high OBD) with the medium group (n = 45) showing mild cognitive dysfunctions (MCD) in memory, language, orientation, and ADL. People with MCD show OBD and BIORISK scores that are significantly different from controls and AD.

Conclusion: Petersen's aMCI criteria cannot be validated and should be replaced by the more restrictive, biologically validated MCD class.

Keywords: Dementia, neurocognition, neuroimmune, oxidative stress, antioxidants, psychiatry, aging

INTRODUCTION

Alzheimer's disease (AD), the major cause of dementia, is a progressive brain disorder characterized by neuroinflammatory and neurodegenerative processes[1-4]. The early phases of AD are characterized by a gradual decline in neurocognitive functions including impairments in episodic and semantic memory and word fluency[5,6]. In the later stages of AD, patients suffer from deficits in memory, language and naming, orientation, executive functions, perceptual-motor functions, attention, and social skills including communication and judgement[6,7]. At that stage, difficulties to perform activities of daily living (ADL) and neuropsychiatric symptoms, such as behavioral dysregulation, irritability and aggression, inertia, and depressive, vegetative, and psychotic symptoms may be evident[7]. The pathophysiology of AD comprises neuroinflammation with astrogliosis, neurofibrillary tangles, accumulation of amyloid plaques, dystrophic neurites with tau protein, and synaptic, neuronal, and neuropil loss[2,3,8,9].

Aging and genetic factors are the most important unmodifiable risk factors for AD, and the apolipoprotein E epsilon 4 (ApoE4) allele is the most widely replicated genetic risk factor of AD[7,10]. Around 40% of AD patients carry the ApoE4 allele, and the risk of AD is increased in E2/E4 (odds ratio = 2.6), E3/E4 (odds ratio = 3.2), and especially E4/E4 (odds ratio = 14.9) carriers[10]. These ApoE genotypes impact the delivery of lipids to cells and amyloid-β deposits and are associated with increased oxidative stress in the brain and a proinflammatory glial response to inflammatory stimuli[11,12], which play a role in synaptic dysfunctions, neuroinflammation, and neurodegeneration[9,12]. In Thai AD patients, we found that ApoE4 carriers have more impairments in tests of semantic an episodic memory, recall, constructional praxis and praxis recall, naming, clock drawing, ADL functions, and Mini Mental State examination, as well as in communication, language, and judgement[7]. Nevertheless, recent research shows that interactions of the ApoE4 genotype with peripheral biomarkers predict greater impairments in semantic and episodic memory and recall, suggesting that such interactions may play a role in the pathophysiology of AD[13]. For example, interactions between the presence of the E4 allele and fasting blood glucose (FBG) and albumin and the cumulative effects of the E4 allele with folic acid, glucose, albumin, and the atherogenic index of plasma (AIP) may increase cognitive deficits in memory and naming and the symptomatic burden in AD[6,13]. Moreover, both hypertension and T2DM may increase risk of AD through immune and oxidative stress-associated mechanisms[14-17]. This is important, as these peripheral biomarkers, hypertension, and T2DM may increase inflammatory and oxidative pathways, thereby aggravating the detrimental effects of the ApoE4 genotypes[6,13]. Nevertheless, no studies have examined whether interactions among those biomarkers, hypertension, and T2DM may impact the neurocognitive as well as behavioral and social dysfunctions in AD.

Another unresolved issue is whether similar interaction patterns between the E4 allele and peripheral biomarkers, hypertension, and T2DM may be observed in patients with amnestic mild cognitive impairment (aMCI). Such associations would support that aMCI is a transition stage between normal aging and AD. aMCI is defined as a decline in memory beyond and above that expected by age and the absence of

dementia symptoms and dysfunctions in ADL[18]. In the classic classification of aMCI, two subtypes were described, namely single-domain aMCI, with isolated impairments in episodic memory, and multiple-domain aMCI, with impairments in episodic memory and one or more other cognitive domains[19]. Individuals with aMCI show an elevated risk to develop AD with a yearly conversion rate from aMCI to AD between 14% and 16%, although some aMCI individuals (8%) may return to the normal state[20]. Interestingly, the ApoE4 allele coupled with an interaction between ApoE4 and FBG is associated with memory deficits in people with aMCI[13].

Nevertheless, aMCI as defined by Petersen's criteria is not a well-modeled entity or nosological class but a heterogenous group of subjects[7]. In fact, there are two main problems with Peterson's criteria: (1) using machine learning techniques such as soft independent modeling of class analogy (SIMCA) performed on neurocognitive test results, we were unable to adequately model the group of people with aMCI using neuropsychological memory tests as modeling variables; and (2) using SIMCA, some aMCI subjects were authenticated as healthy people while others as AD patients[7]. These results show that, using supervised machine learning techniques, Peterson's criteria cannot be validated and, consequently, that unsupervised methods should be used to delineate the subgroup of patients who take up an intermediate position between controls and AD patients.

Recently, we developed a new method, namely nomothetic network analysis, to delineate the causal associations among the causome (e.g., ApoE4 allele), the cognitome (the aggregate of all cognitive dysfunctions), and the phenome including the symptomatome (the aggregate of clinical features) of a neuropsychiatric illnesses[21-23]. This conceptual framework may be analyzed using partial least squares (PLS) analysis to define the significant paths among causome, cognitome, and phenome features, followed by unsupervised learning (e.g., cluster analysis), applied to all features of the causal model, to delineate new, more meaningful subgroups.

Hence, the aims of the present study were: (1) to define the paths from biomarkers (ApoE4, FBG, folate, AIP, albumin, hypertension, and diabetes) and their interactions to the cognitome and phenome of aMCI and AD; and (2) to define and validate the subgroup of aMCI patients who take up an intermediate position between controls and AD patients with respect to cognitive, behavioral, social and biomarker data.

METHODS

Participants

This was a cross-sectional study which included people with AD and aMCI and normal controls, aged 55-90 years and of both sexes. Patients were recruited at the Dementia Clinic, Outpatient Department, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Healthy volunteers were recruited from the same catchment area as the patients, namely Patumwan district, Bangkok province. The normal controls were community senior club members, senior Red Cross volunteers, healthy individuals who visited the Health Check Up Clinic, and normal elderly caregivers of the AD patients who visited the Dementia Clinic. We excluded patient and controls with: (1) abnormal VDRL, HIV, vitamin B12, and thyroid function blood tests; (2) other dementia syndromes, including frontotemporal lobe dementia and vascular dementia; (3) neurologic disorders, including Parkinson's disease, stroke, multiple sclerosis, encephalitis, meningitis, and traumatic brain injury; (4) major psychiatric disorders including major depressive disorder, bipolar disorder, schizophrenia, substance use disorders, and anxiety disorders; and (5) (auto)immune disorders, including rheumatoid arthritis, chronic obstructive pulmonary disease, systemic lupus erythematosus, inflammatory bowel disease, chronic kidney disease, cancer, diabetes type 1, and severe heart disease (Functional Class II or more). Furthermore, controls and patients were excluded when the score on the Thai

Geriatric Depression Scale was > 13 in order to exclude people with a recent depression[7]. We conducted magnetic resonance imaging of the brain in the AD patients to rule out vascular dementia and brain tumors.

Finally, all participants were allocated into three study samples: 61 healthy controls, 60 subjects with aMCI, and 60 AD patients. All participants and guardians of aMCI and AD individuals gave written informed consent prior to participation in this study. The study was conducted according to Thai and international ethics and privacy laws. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No. 359/56), which is in compliance with the International Guideline for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, CIOMS Guidelines, and the International Conference on Harmonization in Good Clinical Practice.

Clinical measurements

Two senior psychiatrists or neurologists experienced in dementia research assessed patients and controls and completed a semi-structured interview to assess clinical history and diagnostic criteria, and they performed neurological and physical examinations, interviewed the close relatives of all participants, and measured the Thai version of Clinical Dementia Rating Scale (CDR)[24]. A senior neuropsychologist specialized in dementia assessed neuropsychological test batteries and the Thai Mental Status Examination (TMSE)[25,26]. The neuropsychologist was blinded from the screening data of the physicians, and the latter were blinded from the assessment results of the neuropsychologist.

We made the diagnosis of AD using the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) diagnostic criteria[27]. Moreover, other inclusion criteria were a TMSE score between 10 and 23 and a CDR score of 1 or 2. aMCI patients were included if they showed subjective memory complaints and when they complied with Peterson's criteria[28]. Subjective memory complaints were assessed using the question "do you feel that your memory had become worse?". Objective memory complaints were established with a CDR score of 0.5 and a CDR memory component score of 0.5. aMCI patients were included when the TMSE score was > 23 and when they were not diagnosed with dementia according to the NINCDS-ADRDA[27]. The healthy controls did not complain of subjective memory, and they showed a TMSE score > 23 and CDR = 0. The diagnoses were discussed by the two physicians for agreement, and, in the case of disagreement, a third opinion from another psychiatrist or neurologist was requested.

The same day, a senior neuropsychologist completed the CERAD Neuropsychological Assessment Battery (CERAD-NP)[7,29] in Thai, using a validated translation. In this study, we used: the Verbal Fluency Test (VFT) to assess semantic memory and cognitive flexibility; the Modified Boston Naming Test (BNT) to assess confrontational word retrieval; the Word List Memory (WLM) to assess episodic memory and learning ability for new verbal information and immediate working memory; WL Recall, Delayed, True Recall (WLRecall) to probe verbal episodic memory and the ability to recall; the WL Recognition Test to assess verbal episodic memory-discriminability or verbal learning recall recognition; and the Constructional Praxis and Recall tests to probe visuoconstructive abilities and later task recall. Moreover, we assessed: (1) C1 or the clinical history items, including memory, language, personality and behavior, orientation for time and place, ADL, social activities, judgment and problem solving, and other cognitive problems; (2) C2 or the ADL Blessed Dementia Scale, Part a (BDS); C3 or the Behavior Rating Scale for Dementia (BRSD) including depressive features, defective self-regulation, irritability/agitation, vegetative features, inertia/apathy, and psychotic features; C4 or the Short Blessed test (orientation-memory-concentration); and C5 or calculation, clock, and expressive language (CCL).

Biomarker assays

APOE genotyping

As described previously[7], we extracted genomic DNA from peripheral blood leukocytes by standard procedures with a DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). Consequently, DNA was amplified by using two primers, 5'-ACAGAATTCGCCCCGGCCTGGTACACAC-3' and 5'-TAAGCTTGGCACGGCTGAAGGA-3'. Each amplification reaction contained 1 μg of leukocyte DNA, 1 pmol/μL of each primer, 10% dimethyl sulfoxide, and 0.025 units/pL of *Taq* polymerase in a final volume of 30:l. Each reaction mixture was heated at 95 °C for 5 min followed by 40 cycles of 95 °C for 60 s, 65 °C for 80 s, and 72 °C for 80 s with a final extension at 72 °C for 7 min. The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, USA) according to the protocols supplied by the manufacturer and shipped for direct sequencing to Macrogen Inc. (Seoul, South Korea). In the statistical analyses, we used an "ApoE4" group, which comprised E4 allele carriers, namely people with the E4/E4 (n = 6), E3/E4 (n = 32), and E2/E4 (n = 5) genotypes[7]. Indeed, one E4 copy (E2/E4 and E3/E4) increases risk for AD and two E4 copies (E4/E4) increase risk considerably[7,10].

Other biomarkers

Fasting blood was sampled between 8:00 and 8:30 a.m. We used 3 mL clotted blood (serum), which was centrifuged at 1000 g for 5 min, to assay biomarkers at the Central Laboratory, Department of Laboratory Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. As explained previously[6,13], we used the Architect C8000 (Abbott Laboratories, Abbott Park, Illinois, USA) to measure the biomarkers. Plasma glucose was measured using A Hexakinase/G-6-PDH technique (inter-assay coefficients of variability of 2.0%). Based on the lipid profile, we computed the AIP index as a z-unit weighted composite score, i.e., z-transformed triglyceride values (zTG) - z high density lipoprotein cholesterol (labeled as zAIP)[6]. In addition to albumin, the present study used total number of white blood cells (WBC) as another indicator of immune activation. Folate levels were measured using electrochemiluminescence immunoassay using the Cobas 6.000 Analyzer (Roche, Germany).

Statistics

We used the analyses of variance to check differences in scale variables between study groups and analyses of contingence tables (χ^2-test) to check associations among categorical variables. We employed multivariate general linear model (GLM) analysis to check the associations between diagnostic classes and clinical and biomarker data while adjusting for age, sex, and education. Tests for between-subject effects were used to check the univariate associations between the classes and clinical and biomarker data. Consequently, we computed GLM model-derived estimated marginal means (SE) after adjusting for age, sex, and education. We used the protected least significant difference to assess pair-wise differences among group means. False discovery rate p-correction was used to correct for multiple comparisons[30]. We used multiple regression analysis to assess the biomarkers that predict latent vector scores while allowing for the effects of age, sex, and education. An automated stepwise method was employed with p-to-enter of 0.05 and a p-to-remove of 0.06. Multivariate normality (Cook's distance and leverage), the R^2 changes, multicollinearity (using the variance inflation factor and tolerance), and homoscedasticity (tested with the White and modified Breusch-Pagan tests) were always checked. Moreover, the regression analysis was performed on 5000 bootstrap samples and the latter results are shown if the results are not concordant. Tests were two-tailed and a P-value of 0.05 was used for statistical significance. Two-step cluster analysis was employed to define clusters of patients based on the cognitome and phenome features. Nearest neighbor analysis was employed to classify subjects based on their feature similarities. All statistical analyses were performed using IBM SPSS windows version 25.

Smart partial least squares (SmartPLS)-SEM analysis[31] was used to assess the causal associations among ApoE4, age, sex, education, and the cognitome and symptomatome of aMCI and AD. We used a multi-step, multiple mediated PLS path model with ApoE4, age, sex, and education as input variables and symptomatome data as output variables, while cognitome data mediated the effects of the input on the output variables. ApoE4, age, sex, and education were entered as single indicator variables. Where possible, we entered the cognitome and symptomatome data as latent vectors extracted from the different tests and clinical scores. When indicator variables could not be combined in latent vectors, they were entered in the analysis as single indicators. Complete SmartPLS analysis was conducted when the outer and inner models complied with specific pre-specified quality criteria: (1) the overall model fit SRMR is < 0.08; (2) the vector loadings are all > 0.666 at $P < 0.001$; (3) the outer model latent vectors show a good construct validity, namely composite reliability > 0.7 and average variance extracted (AVE) > 0.5; and (4) confirmatory tetrad analysis shows that the latent vector models constructed as reflective models are not mis-specified. Complete PLS-SEM analysis performed on 5000 bootstrap samples was used to compute outer model loadings and path coefficients with *P* values and specific indirect and total effects. The predictive power of the model was assessed using blindfolding and PLSpredict with 10-fold cross-validation.

RESULTS

Results of PLS analysis

Figure 1 shows the final PLS model. The symptomatome was structured in four different vectors, namely ADL+OR (BDSa, SBT score, and C1 orientation), BEHAVIOR (the C3 BRSD items comprising depressive features, irritability/agitation, vegetative features, inertia/apathy, and defective self-regulation), MEM+LANG (comprising the C1 clinical history items memory and language), and SOCIAL (including the C1 clinical history items social activities, judgment and problem solving, and other cognitive problems). We also constructed two neurocognitive test latent vectors: (1) a CERAD latent vector comprising the scores on the BNT, VFT, WLM, WLRecall, WLRecognition, Constructional Praxis and Constructional Praxis Recall; and (2) a latent vector comprising calculation, clock drawing, and expressive language (CCL latent vector). In the mediation model, CCL and CERAD mediated the effects of age, ApoE4, and education (entered as single indicator input variables) on the four symptomatome latent vectors. Finally, the four symptomatome latent vectors were used as predictors of the diagnosis (entered as 0, 1 and 2 for controls, aMCI, and AD, respectively). The model displayed in Figure 1 shows an adequate model fit with SRMR = 0.057. The construct reliabilities of all six latent vectors are adequate with all AVE values > 0.542 and all composite reliabilities > 0.755. Moreover, all loadings on the seven latent vectors were all > 0.66 at $P < 0.0001$, and the vectors were not mis-specified as reflective models. Blindfolding showed that the construct cross-validated redundancies of all constructs were adequate. All Q^2 predict scores of the indicators were positive, indicating that they outperform the most naïve benchmark.

We found that 87.7% of the variance in the diagnostic variable was explained by (in ascending order of importance) CERAD, ADL+OR, MEM+LANG, and BEHAVIOR, while CCL and SOCIAL did not have a significant impact. PLS showed that 44.9% of the variance in CERAD could be explained by ApoE4, age, and education, and that the same indicators explained 22.8% of the variance in CCL. Moreover, the effects of CERAD and CCL on the diagnostic spectrum was partially mediated by ADL+OR, BEHAVIOR, and SOCIAL. The specific indirect effects showed that most pathways from the input to the output variables were significant. For example, the effects of ApoE4, age, and education on the diagnosis were mediated by CERAD, or CERAD → ADL+OR, CERAD → BEHAVIOR, or CERAD → MEM+LANG, but not CCL → ADL_OR. The significant specific indirect effects of sex on the diagnosis were mediated by ADL+OR. In addition, all possible total effects were significant with total effects of ApoE4 on CERAD, CCL, ADL+OR, BEHAVIOR, SOCIAL, and MEM+LAN.

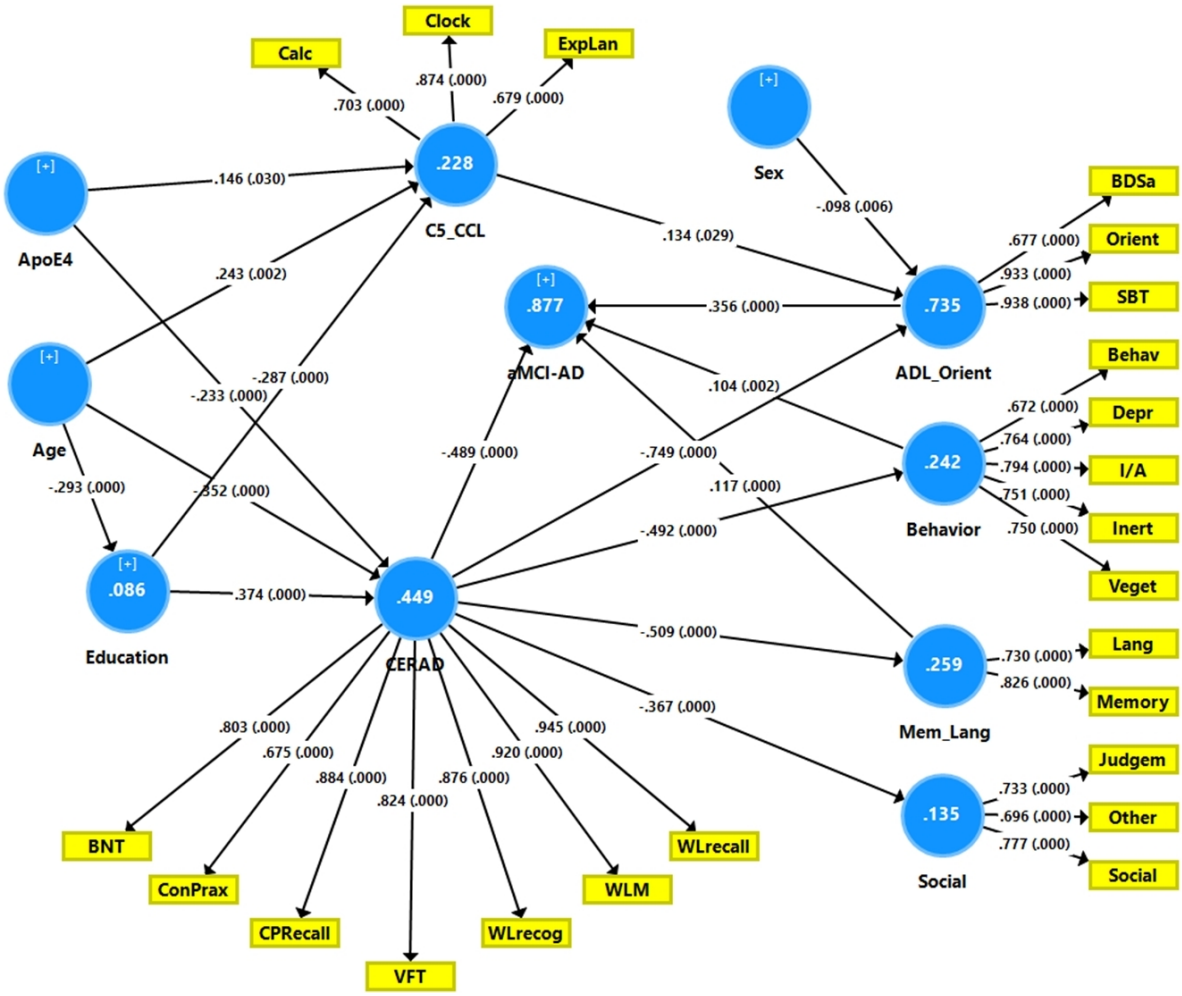

Figure 1. Results of complete partial least squares analysis performed on 5000 bootstrap samples. Path coefficients and loadings (with *P* values) are shown. White figures within the circles indicate percentage of variance explained. The ApoE4 allele, age, sex, and education are input variables and symptomatome data are output variables with cognitive data mediating the effects of the input on the output variables. CCL: calculation (calc), clock, expressive language (ExpLan); CERAD: a latent vector extracted from various CERAD scores including BNT (Boston Naming Test), ConPrax (Constructional Praxis), Recall (CPRecall), VFT (Verbal Fluency Test), WLRecog (Word List Recognition), WLM (Word List Memory), and (WLRecall); ADL_Orient: activities of daily living and orientation, comprising the Blessing Dementia Scale Part a (BDSa), SBT (Short Blessing Test), and C1 orientation; Behavior: Behavior Rating Scale for Dementia subdomains, including depressive features (depr), defective self-regulation (beh), irritability/agitation (I/A), vegetative features (veget), and inertia/apathy (inert). Mem_Lang: C1 clinical history items, including memory and language (Lang); Social: C1 clinical items, including social activities, judgment and problem solving, and other cognitive problems.

Results of nearest neighbor analyses

Two different nearest neighbor analyses were performed to classify subjects as controls, aMCI, or AD patients. The first was conducted using memory scores only, namely WLM, WLRecall, and C1 memory scores (3k, Euclidian distance, training sample of 70%, and a holdout sample of 30%). The classification table shows that many aMCI cases were misclassified as controls in the training (35.7%) and holdout sample (45.4%), yielding a total accuracy of only 68.9% in the holdout sample. The second analysis was conducted using all cognitome and phenome latent vectors extracted by PLS scores (3k, Euclidian distance, training sample of 70%, and a holdout sample of 30%), and this analysis showed 45.0% misclassifications in both the training and holdout samples with many aMCI subjects being allocated to the normal control class.

Construction of an overall burden of disease score and associated subgroups

We computed the latent variable scores of all cognitome and phenome data obtained by PLS and computed an overall composite score indicating overall burden of disease (OBD) computed as: z score of (z CCL + z ADL+OR + z BEHAVIOR + z MEM+LANG + z SOCIAL - z CERAD). Consequently, using a visual binning method (based on inspection of the apparent modes and local minima of the frequency histogram and the results of the two-step cluster analysis described below), we divided the study group into three non-overlapping samples, namely normal, medium, and high OBD (cutoff points were -0.53 and 0.4, respectively). Two-step cluster analysis performed on CCL, CERAD, ADL+OR, MEM_LANG, BEHAVIOR, SOCIAL, and OBD scores in the normal + medium OBD groups retrieved two clusters (based on Akaike's Information criterion) with an adequate silhouette measure of cohesion and separation of 0.57. This cluster solution separated the medium OBD from the normal OBD class. Table 1 shows the association between this new OBD and cluster analysis classification and the classification into HC, aMCI, and AD. There was a highly significant association between both classification systems (χ^2 = 206.97, df = 4, P < 0.001), whereby Group 1 comprised most controls (except 2) + 21 aMCI subjects (this group was labeled "normal OBD group"), Group 2 consisted of 36 aMCI + 2 healthy controls + 7 AD subjects (labeled "medium OBD group"), and Group 3 comprised 53 AD + 3 aMCI subjects (labeled "high OBD group").

Clinical and biological features of the OBD classes

Table 2 shows the features of the three OBD groups formed. Patients with high OBD are somewhat older that the other groups with fewer years of education. There were no differences in the sex ratio or cardiovascular disease frequency among the three study groups. The frequency of hypertension increased as controls → medium OBD → high OBD and that of type 2 diabetes mellitus (T2DM) was higher in both OBD groups than in controls. The frequency of ApoE4 was significantly higher in the high OBD group than in the normal OBD group.

To assess the associations between these groups and the clinical and neuropsychological scores we used multivariate GLM analysis and adjusted for age, sex, and education. There was a highly significant effect of the diagnostic classes (partial eta squared = 0.629; F = 41.01, df = 14/340, P < 0.001) and a very modest effect of education (partial eta squared = 0.082; F = 2.15, df = 7/169, P = 0.041), while sex and years of education did not have significant effects. Tests of parameter estimates show that education was only associated with the CERAD score (inversely, P = 0.005). The CCL, CERAD, ADL+OR, and MEM+LANG latent variable scores increased as normal OBD → medium OBD → high OBD. The BEHAVIOR and SOCIAL scores were significantly increased in the high OBD group as compared with the other two groups. These differences remained significant after FDR p-correction. Table 2 also shows the measurement of blood biomarkers in the three study groups. FBG was significantly higher in the high OBD group than in the normal and medium OBD group. Albumin and folate were significantly lower in the high OBD group than in the normal and medium OBD group, while WBC number and zAIP were significantly increased in the high OBD group as compared with the other two groups. FDR p-correction did not change these results.

We also computed a z unit weighted composite score that comprises all biomarkers (zBiomarkers) as a z ApoE4 + z WBC + z FBG + z AIP - z Alb - zFolate (labeled as zBiomarkers). In addition, we computed another z unit-based composite score as: zBiomarkers + z hypertension + z T2DM (labeled as zBIORISK). Table 2 shows that both indices were significantly different between the three groups and increased as normal OBD → medium OBD → high OBD group. The distance from the medium OBD to the normal OBD group was 0.480 SD, and between the medium and high OBD the distance was 0.743 SDs. Finally, we calculated a composite score as zBIORISK + zOBD score. The distance from the medium OBD to the normal OBD group was 0.641 SDs, and between the medium and high OBD the distance was 1.314 SDs, indicating that the medium OBD group takes up an intermediate position between the normal and high

Table 1. Associations between the classes derived from cluster analysis and the diagnosis of amnestic mild cognitive impairment and Alzheimer's disease

Classes	Normal OBD[A]	Medium OBD[B]	High OBD[C]	Total
HC	59	2	0	61
aMCI	21	36	3	60
AD	0	7	53	60
Total	80	45	56	181

OBD: Overall burden of disease classes (computed based on the results of cognitive, behavioral, and social rating scores); HC: heathy controls; aMCI: amnestic mild cognitive impairment; AD: Alzheimer's disease.

OBD groups, but it is much closer to the normal OBD group than to the high OBD group.

Associations between biomarkers and the OBD indices

Table 3 shows the intercorrelation matrix between the zBiomarker and zBIORISK factors and the clinical and neuropsychological scores. We found that these risk indices were significantly associated with CCL, CERAD, ADL+OR, BEHAVIOR, MEM+LANG, and SOCIAL scores. Table 4 shows the results of multiple regression analysis with the OBD score as dependent variable and the biomarker indices as explanatory variables while allowing for the effects of age, sex, and education. Regression #1 shows that 40.2% of the variance in OBD was explained by the regression on zBIORISK, age, ApoE4 (all positively), and education (inversely). Figure 2 shows the partial regression of the OBD score on the zBIORISK score. We also examined the effects of the biomarker score on the OBD score in restricted samples, namely normal + medium OBD and medium + high OBD groups. Regression #2 shows that, in the restricted study sample of normal + medium OBD subjects, 19.5% of the variance in the OBD score could be explained by zBIORISK and age (both positively) and education (inversely). Regression #3 shows that, in the medium + high OBD group, 17.0% of the variance in the OBD score could be explained by zBiomarkers (positively) and education (inversely).

DISCUSSION

aMCI cannot be validated

The first major finding of this study is that nearest neighbor (supervised learning) and two-step clustering (unsupervised learning) analysis were not able to confirm the existence of aMCI even when using memory scores, which define aMCI. These results corroborate those of our previous report showing that using SIMCA, another supervised learning technique[7], the aMCI subgroup cannot even be modeled using neuropsychological memory tests, which are intended to describe this subgroup[18]. Moreover, using SIMCA, we found that many participants with aMCI were authenticated as controls or as AD patients. Thus, both unsupervised and supervised techniques show that aMCI according to Peterson's criteria is a heterogenous group and, consequently, does not exist as a distinct class. In fact, this is further corroborated by the low diagnostic performance of different neuropsychological tests when discriminating aMCI subjects from controls. These figures show an accuracy of around 70%-80%, as reviewed in[32], where in fact a boostrapped accuracy of > 95% would be needed to obtain a good separation. Using the most adequate machine learning techniques to classify subjects (including support vector machine or neural network analysis) did not improve these figures considerably[32,33].

Mild cognitive dysfunctions as an intermediate class

The second major finding of this study is that we were able to compute a new overall burden of cognitive, social, and behavioral deterioration (OBD) score, which is useful as a severity score and to delineate a reliable subgroup located between controls and AD patients. This OBD index combines the different

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Table 2. Sociodemographic, clinical, and biomarker data of the participants binned into three groups based on the overall burden of disease score and cluster analysis

Variables	Group 1 or normal OBD (*n* = 80)[A]	Group 2 or medium OBD (*n* = 45)[B]	Group 3 or high OBD (*n* = 56)[C]	F/FEPT/χ^2	df	*P*
Age (years)	69.5 ± 6.1[B,C]	75.2 ± 6.9[A,C]	79.0 ± 6.7[A,B]	36.05	2/178	< 0.001
Sex	18/62	11/34	17/39	1.10	2	0.576
Education (years)	12.4 ± 5.0[B,C]	9.5 ± 5.9[A,C]	6.2 ± 5.0[A,B]	23.01	2/178	< 0.001
CVD (N/Y)	75/5	41/4	48/8	2.52	2	0.284
Hypertension (N/Y)	61/19[B,C]	25/20[A,C]	15/41[A,B]	32.68	2	< 0.001
T2DM (N/Y)	72/8[B,C]	33/12[A]	42/14[A]	7.30	2	0.026
Allele E4 (N/Y)	71/9[C]	34/11	33/23[A]	16.19	2	< 0.001
CCL (z scores)*	-0.531 ± 0.091[B,C]	-0.248 ± 0.107[A,C]	0.959 ± 0.110[A,B]	49.27	2/175	< 0.001
CERAD (z scores)*	0.751 ± 0.059[B,C]	0.054 ± 0.069[A,C]	-1.117 ± 0.071[A,B]	167.62	2/175	< 0.001
ADL+OR (z scores)*	-0.751 ± 0.059[B,C]	-0.261 ± 0.069[A,C]	1.282 ± 0.071[A,B]	210.39	2/175	< 0.001
Behavior (z scores)*	-0.460 ± 0.107[C]	-0.221 ± 0.125[C]	0.834 ± 0.129[A,B]	27.18	2/175	< 0.001
Memory+Language (z scores)*	-0.703 ± 0.099[B,C]	0.207 ± 0.117[A,C]	0.838 ± 0.120[A,B]	40.50	2/175	< 0.001
Social (z scores)*	-0.461 ± 0.109[C]	-0.299 ± 0.128[C]	0.900 ± 0.132[A,B]	30.62	2/175	< 0.001
OBD index (z scores)*	-0.819 ± 0.048[C]	-0.196 ± 0.056[C]	1.327 ± 0.057[A,B]	345.26	2/175	< 0.001
FBG (z scores)	-0.247 ± 0.812[C]	0.062 ± 0.927	0.304 ± 0.1.204[A]	5.39	2/178	0.005
Albumin (z scores)	0.201 ± 0.923[C]	0.084 ± 0.896[C]	-0.361± 1.098[A,B]	5.75	2/178	0.004
White blood cells (z scores)	-0.240 ± 0.856[C]	-0.060 ± 1.077[C]	0.391 ± 1.025[A,B]	7.11	2/178	0.001
Folate (z scores)	0.242 ± 0.901[C]	0.111 ± 0.996[C]	-0.436 ± 1.012[A,B]	8.61	2/178	< 0.001
zAIP (z scores)	-0.184 ± 0.999[C]	-0.092 ± 1.020[C]	0.337 ± 0.914[A,B]	4.94	2/178	0.008
zBiomarkers (z scores)	-0.453 ± 0.730[B,C]	-0.089 ± 0.885[A,C]	0.720 ± 1.023[A,B]	30.38	2/178	< 0.001
zBIORISK	-0.498 ± 0.708[B,C]	-0.018 ± 0.889[A,C]	0.725 ± 1.011[A,B]	33.56	2/178	< 0.001
zBIORISK+OBD	-0.761 ± 0.432[B,C]	-0.123 ± 0.522[A,C]	1.191 ± 0.694[A,B]	212.06	2/178	< 0.001

All variables are shown as mean (SD) except * marginal estimated mean (SE) after covarying for age, sex, and education. [A,B,C]Pairwise comparisons among treatment means. CVD: Cardio-vascular disease; T2DM: type 2 diabetes mellitus; CCL: calculation, clock, expressive language; CERAD: a latent vector extracted from various CERAD scores including memory, naming, and praxis; ADL+OR: activities of daily living and orientation; OBD: overall burden of cognitive, behavioral, and social deterioration; FBG: fasting blood glucose; zAIP: atherogenic index of plasma. zBiomarkers: z unit weighted composite score computed as z ApoE4 + z white blood cells + z FBG + z AIP - z Albumin - z Folate. zBIORISK: computed as zBiomarkers + z hypertension + z T2DM.

cognitome (CCL and CERAD) and phenome (ADL+OR; BEHAVIOR, MEM+LANG, and SOCIAL) domains of the dementia spectrum into one integrated index of overall burden of disease and, therefore, ranks subjects along a continuum from a normal condition to severe dementia. Using a visual binning method performed on the OBD scores, we divided the study sample into three classes, namely subjects with normal, high, and medium OBD scores and validated the medium OBD class using two-step cluster analysis. This medium OBD group is less inclusive than aMCI and is characterized by impairments in

Table 3. Intercorrelation matrix between biomarker and clinical and cognitive scores

Variables	zBiomarkers	zBIORISK
CCL	0.347	0.352
CERAD	-0.492	-0.499
BEHAVIOR	0.298	0.302
MEM+LANG	0.279	0.306
SOCIAL	0.396	0.419
ADL+OR	0.425	0.465
OBD score	0.475	0.500

All results of Spearman correlation analyses; all $P < 0.001$ ($n = 181$). CCL: Calculation, clock and expressive language score; CERAD: a latent vector extracted from various CERAD scores; CCL: latent vector (LV) extracted from calculation, clock, expressive language; CERAD: a latent vector extracted from various CERAD scores including Boston Naming Test, Constructional Praxis and Recall, Verbal Fluency Test, Word List Recognition, Word List Memory, and Word List Recall; BEHAVIOR: LV extracted from five Behavior Rating Scale for Dementia subdomains, namely depressive features, defective self-regulation, irritability/agitation, vegetative features, and inertia/apathy; MEM+LANG: LV extracted from the C1 clinical history items memory and language; SOCIAL: LV extracted from three C1 clinical items, namely social activities, judgment and problem solving, and other cognitive problems; ADL+OR: LV extracted from the Blessing Dementia Scale Part a, Short Blessing Test, and the C1 item of orientation; OBD: overall burden of cognitive, behavioral, and social deterioration; zBiomarkers: z unit weighted composite score computed as z ApoE4 + z White blood cells + z Fasting Blood Glucose + z Atherogenic Index of Plasma - z Albumin - z Folate; zBIORISK: computed as zBiomarkers + z hypertension + z type 2 diabetes mellitus.

Table 4. Results of multiple regression analyses with the overall burden of cognitive, behavioral, and social deterioration score as dependent variable and biomarker scores as explanatory variables

Dependent variables	Explanatory variables	β	*t*	*P*	F model	df	*P*	R^2
#1. OBD in all	**Model**				29.54	4/176	< 0.001	0.402
	zBIORISK	0.261	3.72	< 0.001				
	Education	-0.256	-4.17	< 0.001				
	Age	0.260	3.87	< 0.001				
	ApoE4	0.135	2.11	0.036				
#2. OBD in normal and medium OBD groups	**Model**				9.43	3/117	< 0.001	0.195
	zBIORISK	0.193	2.05	0.043				
	Education	-0.227	-2.70	0.008				
	Age	0.225	2.41	0.017				
#3. OBD in medium and high OBD groups	zBiomarkers	0.340	3.67	< 0.001	10.01	2/98	< 0.001	0.170
	Education	-0.188	-2.19	0.031				

zBiomarkers: z unit weighted composite score computed as z ApoE4 + z White blood cells + z Fasting Blood Glucose + z Atherogenic Index of Plasma - z Albumin - z Folate; zBIORISK: computed as zBiomarkers + z hypertension + z type 2 diabetes mellitus; ApoE4: any of the E2/E4, E3/E4, or E4/E4 genotypes.

CERAD, CCL, ADL+OR, and MEM+LANG, and, therefore, we propose to name this group Mild Cognitive Dysfunctions (MCD) including in memory, ADL, language, and orientation. Peterson's criteria[18], on the other hand, stress the absence of ADL dysfunctions in aMCI. As such, the features of MCD differ from those of single-domain aMCI and of multiple-domain aMCI, which was thought to be characterized by deficits in episodic memory and one or more other cognitive domains[19]. Moreover, the MCD criteria do not correspond with those of mild behavioral impairment (MBI), which is characterized by persistent behavioral symptoms in late life and is thought to constitute a risk for neurodegenerative disease[34,35]. Indeed, in our study, the MCD group did not display any behavioral or social dysfunctions.

Biomarkers in MCD

The third major finding of this study is that ApoE4 significantly predicted all cognitome (CCL and CERAD)

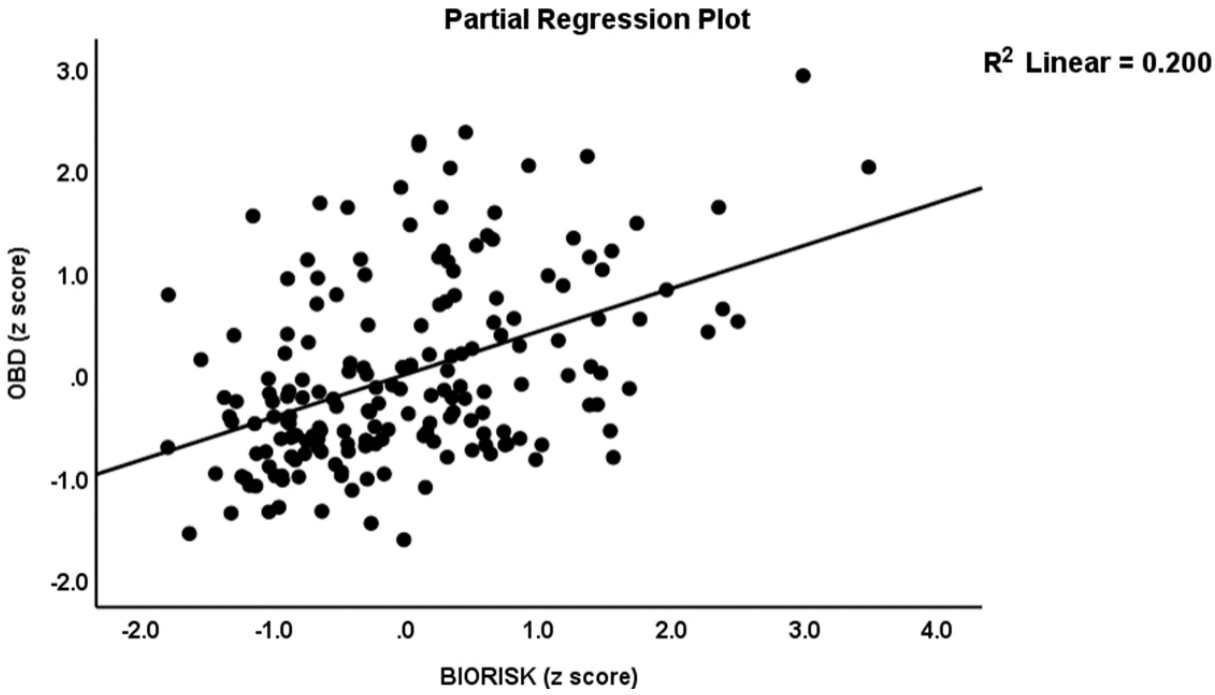

Figure 2. Partial regression of the overall burden of cognitive, behavioral, and social deterioration (OBD) on the BIORISK index. The OBD index was computed using cognitive, behavioral, and social symptoms of the CERAD. The BIORISK score was computed using the ApoE genotypes, serum folate, albumin, white blood cells, fasting blood glucose, atherogenic index of plasma, type 2 diabetes mellitus, and hypertension.

and phenome (ADL+OR; BEHAVIOR, MEM+LANG, and SOCIAL) domains of the dementia spectrum, and that the impact of the ApoE4 allele could be improved by constructing a new composite score reflecting the interactions between the ApoE4 allele, folate, FBG, albumin, WBC, and AIP and comorbid illness including hypertension and T2DM. This biomarker score externally validated the continuous OBD score and the MCD class: (1) people with MCD show higher biomarker scores than controls; and (2) the transitions of controls to MCD and from the latter to AD are both associated with increasing biomarker scores. Phrased differently, an increased impact of interactions among factors which confer risk towards increased glucotoxicity (ApoE4 FBG T2DM), atherogenicity (ApoE4 AIP hypertension), inflammatory responses (ApoE4 albumin WBC), and oxidative stress (ApoE4 folate albumin) underpin both MCD and AD. Since the same biomarker score is associated with AD as well as with MCD, we may conclude that people with MCD probably show an increased risk to develop AD. It is known that individuals with aMCI display an increased risk to develop AD with a conversion rate from aMCI to AD of 14-16.5%[20]. Future research should examine how many MCD subjects show the expected conversion rate to develop AD.

Limitations

The results of this study should be interpreted with regard to the limitations. It would have been more interesting if we had assayed brain imaging biomarkers including the connectome and neuro-immune biomarkers which are known to play a role in AD and cognitive deterioration including levels of neurotoxic cytokines and chemokines, oxidative stress biomarkers, and more specific antioxidants[9,36]. In addition, inclusion of amyloid and tau burden pathology coupled with ApoE4 genetic status could strengthen our mathematical models[37]. The results of the present study merit replication using a larger study sample.

Conclusions

Both supervised and unsupervised learning techniques show that aMCI is a heterogenous class and not a viable entity. In this study, we constructed two z unit-based composite scores: the first reflecting overall burden of cognitive, social and behavioral deterioration (OBD) and the second reflecting the interactions between ApoE4 and other biomarker risk factors, hypertension, and T2DM. The OBS score may be used to assess severity of OBD and AD and classify MCD subjects. The latter show increased biomarker scores which significantly differ from controls and AD patients; therefore, patients with MCD may be at increased risk to develop dementia.

DECLARATIONS

Authors' contributions

Contributed significantly to the paper and approved the final version: Maes M, Tangwongchai S

Availability of data and materials

The dataset generated during and/or analyzed during the current study will be available from the corresponding author upon reasonable request and once the dataset has been fully exploited by the authors.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The study was conducted according to Thai and international ethics and privacy laws. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No 359/56), which is in compliance with the International Guideline for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Consent for publication

All participants and guardians of aMCI and AD individuals gave written informed consent prior to participation in this study.

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Ageing and Neurodegenerative Diseases

Perspective

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Interplay among norepinephrine, NOX2, and neuroinflammation: key players in Parkinson's disease and prime targets for therapies

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Abstract

The role of norepinephrine (NE) in the pathogenesis of Parkinson's disease (PD) has not been well investigated until recently. The purpose of this perspective article is to review evidence supporting the idea that dysfunction of the locus coeruleus (LC)/NE system in the brain may be fundamentally linked to the pathogenesis of PD. Compelling evidence demonstrates that loss of NE neurons in the LC is sufficient to initiate chronic neuroinflammation, resulting in a progressive and sequential loss of neuronal populations in the brain. This article summarizes the critical role of both microglial and neuronal NADPH oxidase 2 (NOX2), the superoxide and reactive oxygen species generating enzyme, as an important regulator of chronic neuroinflammation. Moreover, NOX2 inhibitors show high efficacy in halting chronic neuroinflammation, oxidative damage, and neurodegeneration in several animal PD models. This line of research offers a promising disease-modifying therapeutic strategy for PD.

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Keywords: Parkinson's diseases, progressive neurodegeneration, chronic neuroinflammation, locus coeruleus, noradrenergic system, motor/nonmotor symptoms

INTRODUCTION

Parkinson's disease (PD) is a neurological disorder characterized by progressive neurodegeneration in the nigrostriatal system, resulting in the development of progressive movement disorders[1]. Pathological examination revealed cytoplasmic inclusions known as Lewy bodies or Lewy neurites in the survival dopaminergic (DA) neurons[2,3]. About 15% of PD cases occur in familial clusters at early age[4], which are attributed to mutations in genes, including parkin, leucine-rich repeat kinase 2, and α-synuclein[5]. By contrast, the remaining PD cases are sporadic and may represent the final outcome of a complex set of interactions among the innate vulnerability of DA system, genetic predisposition, and environmental toxins exposure[6]. Exposures to infectious agents, pesticides, or heavy metals in humans increase the risk of acquiring PD[7-13]. We and others have proposed that exposures to these risk factors trigger neuroinflammation, which plays a key role in the pathogenesis of PD[14]. However, this concept has not been proved until recently[15,16].

Microglia and astroglia are the two major types of glial cells involved in the initiation and maintenance of neuroinflammation. Microglia, the resident macrophages in the brain[17], play critical roles in the programmed elimination of neural cells in the early stage of neuronal development[18,19]. As the brain's main immune cells, microglia can rapidly be activated in response to brain injuries and immunological stimuli[20-23]. Activated microglia undergo morphological and functional changes[20] and increase the expression of many surface molecules[24,25]. Activated microglia release a variety of immune factors to recruit more cells and phagocytize foreign substances. In normal physiological conditions, microglia exerts beneficial functions in immune surveillance and depletion of noxious stimuli. By contrast, in pathological conditions, such as chronic inflammation in the brain, microglia can cause neurotoxicity and significantly lead to neurodegeneration.

Different from microglia, astroglia are not derived from immune cell lineage, but they are essential to the integrity and function of the brain[26]. Besides serving as an component of the blood-brain barrier (BBB), astroglia provide physical support and nutrition to neurons, buffer excess neurotransmitters, and maintain ionic homeostasis[26]. Astroglia also become activated under immunologic challenges or brain injuries[27,28]. Activated astroglia secrete a host of neurotrophic factors, such as BDNF, GDNF, and NGF[29,30], which are crucial for the survival of neurons. It has been reported that many anticonvulsant drugs exert potent neuroprotection through astroglia-derived neurotrophic factors[31]. These findings suggest that astroglia are promising targets for developing novel therapies for PD.

Interactions among microglia and astroglia are an important yet not fully studied area. It was found that, in response to immunologic challenges, activation of astroglia often depends on the presence of microglia. Secreted immune factors from prior activated microglia can act and turn astroglia into different phenotypes depending on the immune conditions. In physiological condition, increased release of neurotrophic factors, such as GDNF, BDNF, and NGF, benefits neuronal survival[32,33]. By contrast, neurotoxic reactive astrocytes (A1 astroglia) induced by activated microglia can exaggerate neurotoxicity in pathological condition[34]. Since the role of astroglia in inflammation-related neurodegeneration is less well-documented, this review mainly focuses on the role of microglia in neuroinflammation and neurodegeneration.

Scope of this article

Recent research revealed that low-grade, chronic neuroinflammation is a key to cause progressive neurodegeneration[35,36]. However, the detailed mechanisms involved in the onset and maintenance of chronic neuroinflammation and related neurodegeneration still require additional studies. Emerging evidence suggests critical roles of central norepinephrine (NE) in the pathogenesis of disease progression and manifestations of a variety of nonmotor dysfunctions in PD patients. Thus, this perspective article focuses on the following three aspects.

Neuroinflammation-based rodent PD models

We review several toxin-elicited PD models, which show some of the cardinal characteristics of observed in PD patients, such as chronic neuroinflammation, sequential neurodegeneration, and progressive motor and nonmotor dysfunction.

Roles of central NE in neuroinflammation

Based on common features observed from inflammation-based animal models, we discuss immune factors involved in the initiation and maintenance of low-grade neuroinflammation. The possibility that the loss of locus coeruleus-norepinephrine (LC/NE) neurons may be the focal initiating point in producing a similar pattern of progressive caudal-rostral degeneration by various toxins is evaluated. Furthermore, cellular and molecular mechanisms underlying chronic neuroinflammation-induced progressive neurodegeneration are discussed.

Molecular mechanisms of anti-inflammatory and neuroprotective functions of NE

Anti-inflammatory therapy for neurodegenerative diseases has been emerging as a promising disease-modifying therapeutic strategy. We review the current status in the development of PD therapies by focusing on drugs that affect the NE system.

NEUROINFLAMMATION-BASED RODENT PD MODELS

Disease progression in PD patients

One of the cardinal characteristics of PD is the progressive nature. However, the mechanism of PD progression remains unclear. Currently, PD therapies are limited to symptoms relief, while disease-modifying therapies aimed at stopping PD progression are still lacking. The understanding of PD progression has been greatly facilitated by both basic and clinical research. Braak's group was the first to document a caudal-rostral pattern of disease progression[37]. In PD patients, neuronal loss starts from the lower brain (raphe nucleus, LC, and olfactory bulb) and gradually affects the higher centers of the brain[38]. Further studies uncovered that peripheral inflammation occurs years before PD patients show movement dysfunction. The proposed route of disease progression originating from the gut and spreading to the brain fits well with the symptom progression of PD patients[39]. Before symptoms of movement disorder are observed, gut dysfunction, such as constipation and other premotor disorders, including smell loss, sleep disorder, and other autonomic dysfunction, are often found in patients with PD. Recently, creating animal models mimicking the pattern of neurodegeneration observed in PD patients and investigating its underlying mechanisms has become a widely pursued research area.

Role of neuroinflammation in disease progression

Accumulating evidence strongly indicates that brain inflammation plays a critical role in progressive neurodegeneration. Both gene-mutated and toxin-induced animal PD models have been generated to investigate neurodegenerative pattern and associated motor and nonmotor behavioral changes. This perspective article focuses on only a few commonly accepted toxin-elicited animal PD models, which are inflammation based and show some of the cardinal progressive features observed in PD patients.

LPS model

Peripheral inflammation induces neuroinflammation and neurodegeneration

Most rodent PD models, including those generated by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or 6-hydroxydopamine, display acute toxicity within days but fail to recapitulate the delayed, progressive pattern of DA neurodegeneration. To investigate whether chronic neuroinflammation plays a role in the progressive neurodegeneration of PD, several environmental risk factors implicated in the pathogenesis of PD (e.g., pesticides, herbicides, and infectious agents) and whether they could recapitulate the delayed, progressive nature of PD have been determined in rodents by our lab and others[35,40,41]. Gram-negative bacterial endotoxin LPS is one of the commonly used toxins. Following a systemic injection of lipopolysaccharide (LPS, 5 mg/kg), mice show delayed, progressive neurodegeneration of DA neurons[35]. Further studies indicate that this model not only provides an excellent tool for studying the role of neuroinflammation-related neuronal damage but also serves as a useful platform for exploring drug therapies in PD. Clinically, the relevance of the LPS model for PD is supported by several case reports, in which a significant correlation between infections and the risk of developing PD was found[42].

To investigate the role of gene-environment interactions in the etiology of PD, we created an accelerated rodent PD model by LPS in transgenic mice over-expressing mutant human α-synuclein (A53T). After a single intraperitoneal injection of LPS (1 mg/kg) in seven-month-old male mutant *A53T* mice (*Tg* mice) and wild type controls (*WT* mice), the delayed, progressive degeneration of nigral DA neurons was observed in *Tg* mice, but not in *WT* mice[43]. After five months of LPS treatment, Tg mice lost more than half of their nigral DA neurons, while the striatal TH levels were reduced by a comparable degree. By contrast, LPS-induced neuronal damage was not observed in *WT* mice or saline-injected *Tg* mice. These results demonstrate synergistic neurotoxicity of LPS and A53T α-synuclein overexpression, thus strongly indicating the critical role of gene-environment interactions in PD. Selective DA degeneration was assessed by immunofluorescence double-labeling with antibodies against TH and Neu-N[43]. An about 52% decrease in nigral DA neurons was found in LPS-injected *Tg* mice, whereas only 9.2% of non-DA neurons were lost. Collectively, this two-hit PD model recapitulated the signature lesion of PD by its chronic, progressive, and selective neurodegeneration of nigral DA neurons.

LPS-elicited chronic neuroinflammation exerts progressive ascending neurodegeneration and behavioral changes

The involvement of neuroinflammation in the pathogenesis of PD was identified decades ago. Positron emission tomography (PET) imaging reveals prominent and heterogeneous neuroinflammation in the brains of patients with PD[44,45]. Strong evidence indicates that LPS-induced chronic neuroinflammation is sufficient to not only induce nigral DA neurodegeneration[35,46,47] but also drive progressive loss of other vulnerable neuronal populations outside the basal ganglia. Mechanistically, LPS-generated sub-lethal septicemia in the periphery is able to activate microglia, resulting in low-grade chronic neuroinflammation in the brain for the remaining lifetime of the mice[35]. The pattern of delayed neurodegeneration in this model is dissimilar to that of the intracranial LPS model that produces acute neurodegeneration. Chronic neuroinflammation elicited by systemic LPS injection enables steady production of cytotoxic factors to damage bystander neurons. In turn, damaged/dying neurons can re-active neighboring microglia through the release of danger-associated molecular pattern (DAMP), forming a self-propelling cycle that eventually leads to sustained neuronal damage[48] .

How does a single intraperitoneal injection of LPS induce long-lasting brain inflammation and progressive neuronal loss?

An intriguing question arises: why can a single injection of LPS produce such robust and long-lasting effects in the brain, since the half-life of LPS in mouse blood is only approximately 12 h[49]? It is well-documented

that, under the physiological conditions, very minimal LPS can enter to the brain due to the poor passage through BBB[50]. Therefore, LPS-induced neuroinflammation appears to be an indirect effect. Studies showed that a single intraperitoneal LPS injection initially produced large amounts of proinflammatory cytokines or chemokines from Kupffer cells, the resident macrophage-like cells in the liver[51]. We found that the levels of cytokines in blood were greatly elevated at early times but declined to basal levels by 6-9 h. Remarkably, proinflammatory levels and microglial activation sustained in the brain for up to 10 months. Further mechanistic studies revealed that blood immune factors can pass through BBB[52]. After entering to brain parenchyma, these proinflammatory factors can activate microglia to continually produce more cytokines, reactive oxygen, and nitrogen species and other cytotoxic factors[35]. These microglia-generated toxic factors cause neuronal damage to release DAMPs, which further reactivate microglia. Through this process, a vicious cycle is formed to maintain neuroinflammation and cause additional neuronal loss [Figure 1].

To further investigate the mechanism of transition of chronic neuroinflammation from the periphery to the brain, mice were administered with TNFα peripherally. The results show that both TNFα and LPS injections elevated the production proinflammatory factors (TNFα, MCP-1, and IL-1β) in the brain. Further, mice deficient in TNFR1/R22/2 receptors failed to show brain neuroinflammation in response to LPS and TNFα challenges, supporting that TNFα was one of the critical factors in bridging inflammation from the periphery to the CNS. Long-lasting and enhanced microglial activation, indicated by the immunohistochemical analysis of brain sections with anti-Iba-1 or anti-CD11b antibodies, was observed in brain regions, such as the SN, hippocampus, and motor cortex. The LPS-elicited long-lasting inflammatory and neurotoxic effects in the brain were consistent with previous findings. Exposed to MPTP, a selective DA neurotoxicant, in humans[53] and monkeys[54], led to sustained microglial activation up to years after exposure. In addition, in utero LPS exposure during a critical window of development (E11) rendered a 30% loss of nigral DA neurons in offspring at the age of seven months[55]. Taken together, these findings suggest that early or brief exposure to toxins/toxicants can induce chronic, self-propelling neuroinflammation and lead to progressive neurodegeneration.

Rotenone model

Rotenone, a previously widely used pesticide, reproduces Parkinsonism associated with increased risk for PD. Since the first publication by Greenamyre *et al.*[8], rotenone has been commonly used as a tool to create a rodent PD model[8]. Chronic rotenone exposure in rodents induces key features of Parkinsonism[56]. Mechanistically, rotenone is believed to impair mitochondrial complex I[57,58] and microtubule-based transport of neurotransmitter vesicles[59,60]. Although the role of mitochondrial complex I deficits has been demonstrated in rotenone-induced Parkinsonism[8,56], inhibition of mitochondrial complex I appears not to be the only mechanism for rotenone-induced DA degeneration[61]. A mouse strain lacking functional Ndufs4, a gene encoding a subunit required for complete assembly and function of complex I, has been used to further address this issue. Genetic ablation of Ndufs4 gene suppressed complex I activity but did not affect DA neuron survival in midbrain cultures prepared from *E14* mice[61].

The involvement of microglia in mediating rotenone-elicited neurotoxicity has also been reported. In midbrain neuron and glia cultures, rotenone showed much higher potency in reducing the survival of DA neurons than that in neuron-enriched cultures[62]. Further studies revealed that microglial NADPH oxidase 2 (NOX2)-derived superoxide markedly exacerbated DA degeneration in rotenone-treated cultures[63], suggesting that microglial NOX2 is an alternative target of rotenone. This finding was further confirmed by a study showing that rotenone directly interacted with the catalytic gp91phox subunit of NOX2[64].

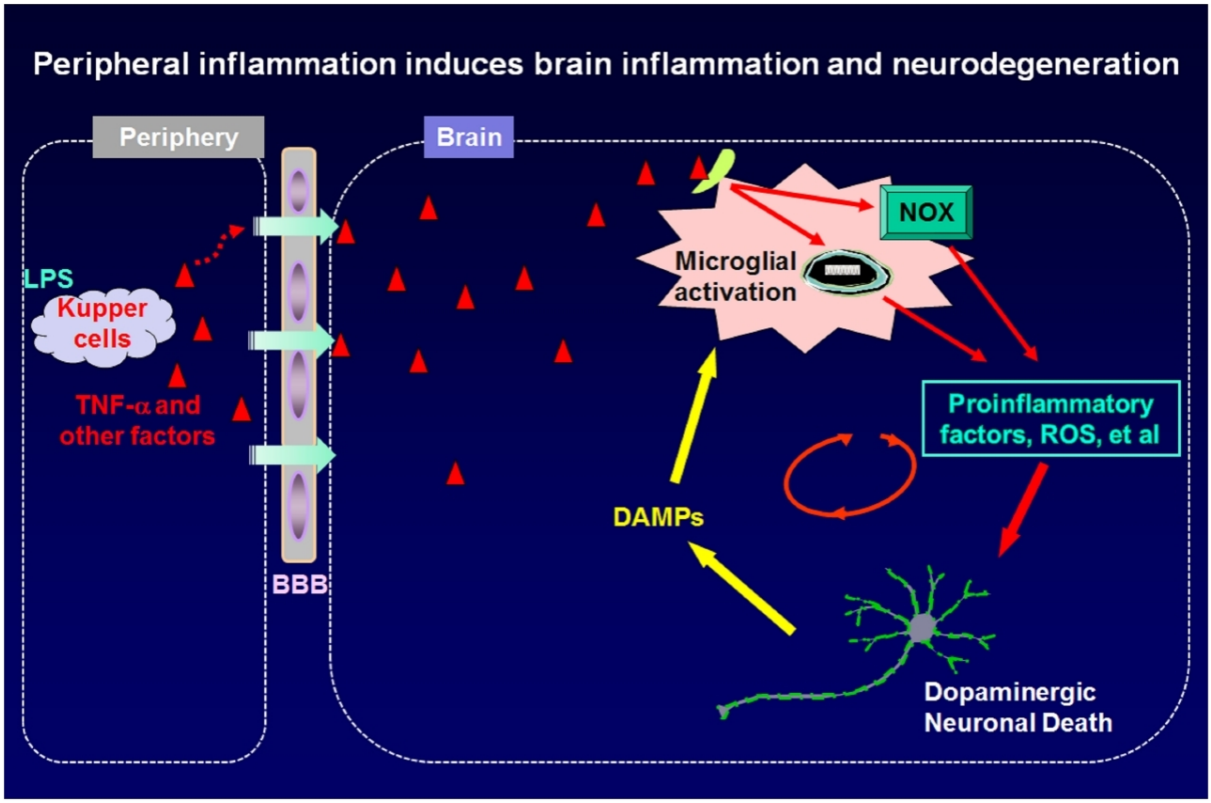

Figure 1. How does a single ip injection of LPS induce long-lasting brain inflammation and produce progressive neuronal loss? LPS reaches the liver via the portal vein circulation and causes it to secrete large amounts of various cytokines, such as TNFα. Some of these cytokines can pass through BBB by receptor-mediated mechanisms to activate microglia and produce additional proinflammatory factors to cause neuron damage. DAMP released from damaged neurons further reactivate microglia to from a self-propelling vicious cycle to maintain chronic neuroinflammation and lead to neurodegeneration. (This figure was modified from our previous paper with permission[136]).

The involvement of microglia in generating NOX2-dependent superoxide in rotenone-treated neuron-glial cultures further suggests a critical role of neuroinflammation in rotenone-induced neurotoxicity. Indeed, a recent report indicated that daily intraperitoneal injections of rotenone for three weeks produce microglia-dependent neuroinflammation in mouse brain[65,66]. Moreover, not only was neuronal loss observed in the SN area, but it also showed a greater loss of LC/NE neurons[65]. Mechanistic studies unraveled that the integrin Mac1/NOX2 complex is a major pathway coupling the production of superoxide and neuroinflammation in rotenone-treated mice[65]. These results provide a novel insight into the pathogenesis of rotenone-induced neurodegeneration. However, how the microglial NOX2 activation is related to the inhibition of mitochondria dysfunction in the rotenone PD model is an interesting but not yet studied question.

Paraquat/maneb model

Paraquat and maneb, two pesticides used in agriculture, are commonly used in many of the same crops. Epidemiological studies revealed an increased risk of PD in human when exposed to combined paraquat and maneb compared with either alone. Paraquat and maneb cotreatment has been widely employed to model PD in rodents. Systemic administration of combined paraquat and maneb led to synergistic damage to nigrostriatal DA neurons and reduction of motor activities in mice[41]. In addition to the DA system, paraquat and maneb co-exposure also damaged neurons in other brain regions and followed a time-dependent ascending neurodegenerative pattern. We recently reported that paraquat and maneb co-treated

mice displayed loss of LC/NE and nigral DA neurons at four weeks after exposure, which was two weeks earlier than that of hippocampal and cortical neurodegeneration[40,67,68]. Consistent with sequential neurodegeneration, paraquat and maneb co-exposure induced gait abnormality and cognitive decline in mice at four and six weeks after treatment, respectively[40,67]. Interestingly, inhibition of microglial activation and production of inflammatory factors by targeting CD11b, the α-chain of Mac-1, or NOX2 significantly mitigated combined paraquat- and maneb-induced neurodegeneration and behavioral abnormalities in mice[40,68,69]. Furthermore, attenuated neuronal damage in paraquat- and maneb-treated mice was also observed once these mice were co-administered with taurine, a major intracellular free β-amino acid with potent anti-inflammatory capacity[67,70,71]. Altogether, these findings suggest that microglia-mediated neuroinflammation contributes to progressive neurodegeneration in this two-pesticide-induced mouse PD model.

NE DYSFUNCTION PLAYS A KEY ROLE IN THE PATHOGENESIS OF PD

Chronic neuroinflammation and progressive neurodegeneration can be generated by various toxins with different modes of action

Animal PD models described in previous section are generated by a variety of toxins with different chemical structures and modes of action. In general, neuroinflammation can be generated by two ways: (1) agents that are infectious, such as microorganism or endotoxins; and (2) chemicals that are not infectious, such as rotenone, paraquat/maneb, or DSP-4, a selective NE neurotoxicant (see below). Despite their differences in initiating neuroinflammation, these toxins somehow produced a similar pattern of neurodegeneration. The pattern of ascending caudal-rostral neurodegeneration generated by a single systemic injection of LPS or DSP-4, (or repeated injections of rotenone) is of the utmost importance for two reasons: (1) it resembles the pattern of neurodegeneration observed in PD patients; and (2) it indicates that a common mechanism is operative to drive a similar pattern of neurodegeneration produced by various toxins, even if they are different in chemical structures and modes of action. Elucidation of this common pathway would greatly advance our understanding of the etiology and pathogenesis of PD. Therefore, rodent PD models generated by LPS (infectious) or DSP-4 (non-infectious) could be useful to investigate possible mechanisms underlying the similar ascending sequential pattern of neurodegeneration induced under different pathological conditions.

Loss of LC/NE neurons is the focal point in producing similar patterns of progressive caudal-rostral degeneration by various toxins

As mentioned above, despite high chemical disparity and toxicological actions, exposure to various toxins/toxicants produces similar patterns of neurodegeneration in mouse brain. Immunochemical analysis reveals a sequential caudal-rostral fashion: neuronal degeneration is first found in the brain stem region, such as LC, followed by neurons in the SN and thalamus, and lastly observed in the hippocampus and cortical regions[36,65,67,72]. Based on these observations, as well as our previous reports indicating anti-inflammatory and neuroprotective functions of NE[73], a logical hypothesis was proposed that loss of LC/NE neurons may be the critical focal point for producing similar patterns of progressive caudal-rostral degeneration by various toxins. Recent progress in this area of research has greatly advanced our understanding of the roles of NE in neurodegenerative diseases, particularly in PD. We review evidence supporting this hypothesis and discuss potential clinical implications of NE dysfunction in PD below.

NE deletion by DSP-4 elicits progressive neurodegeneration

The early loss of LC/NE neurons induced by LPS suggests a possibility that depletion of central NE is a key for progressive neurodegeneration in this neuroinflammatory PD mouse model and even possibly in PD patients. To test this hypothesis, the NE-depleting toxin DSP-4 was used. A single injection of DSP-4 (50 mg/kg; ip) reduced tissue levels of NE (ranging from 55% to 80%) one day after injection in NE-innervated

regions, such as the midbrain, motor cortex, and hippocampus. Brain NE levels remained significantly reduced for up to four months, but they slowly returned to normal by 10 months post injection. Depletion of brain NE levels was accompanied by a time-dependent sequential loss of neurons: as expected, a more than 60% decrease in LC neurons was found one day after DSP-4 treatment. Time-dependent decreases in nigral DA neurons were observed at 4, 7, and 10 months after DSP-4 injection, in comparison to age-matched vehicle controls[36,72]. DSP-4 also led to reduction of Neu-N-positive neurons in the motor cortex and hippocampus, but not in caudate/putamen and ventral tegmentum area 10 months later [Figure 2]. DSP-4-induced neurodegeneration was accompanied by decreased metabolism of glucose detected by PET imaging with [18F]-FDG. The reduced glucose levels were observed in the olfactory bulb, thalamus, hindbrain, midbrain, hippocampus, and across all cerebral cortices at 10 months in DSP-4 injected mice[36], implicating putative neurodegeneration in these brain regions. Again, it is interesting to note that no change of glucose utilization was observed in the cerebellum or the caudate/putamen.

One salient finding of these studies is that the pattern of neurodegeneration in both LPS and DSP-4 models approximate the spatiotemporal progression of neuronal loss in PD. Following the degeneration of LC/NE neurons, both models show significant loss of DA neurons in the SN, yet without affecting DA-neurons in the VTA region. Cortical[74,75] and hippocampal atrophy[76,77], which are often observed in the late-stages of PD, were also found months after LPS or DSP-4 injection. In agreement with neurocircuit degeneration, both LPS- and DSP-4-injected mice displayed behavioral dysfunction, including motor deficits[35] and a variety of nonmotor phenotypes[72] [Figure 2].

These findings approximate the neurodegeneration found in PD patients. The selective neurodegeneration pattern revealed a strong correlation between the concentration of NE and the vulnerability of the intrinsic neurons in LC/NE neuron-innervated regions in response to different toxins/toxicants, such as LPS, DSP-4, rotenone, paraquat, *etc*.[36,65,67,72]. Together, these findings strongly suggest that loss of LC/NE neurons play a pivotal role in producing a similar pattern of progressive caudal-rostral degeneration.

Comparison of LPS, DSP-4, rotenone, and paraquat/maneb models

Different toxins produce neurodegeneration with distinct modes of action. However, based on the initial cell types targeted, most toxins used for modeling PD can be generally classified into three groups.

Cell non-autonomous mechanism

Pathogen associated molecular pattern agents such as microorganisms, endotoxins, or proinflammatory cytokines belong to this class. The primary target cells are microglia in the CNS. Upon the activation of microglia, large amounts of cytokines are released and produce a high degree of acute neuroinflammation to combat the infectious agents. However, over-production of immune factors also causes collateral bystander neuronal damage. Subsequent release of DAMP substances from injured neurons in turn triggers reactive microgliosis through the activation of the MAC-1 receptor, which further activates microglial NOX2, increases the production of superoxide/ROS, and causes additional inflammation and neuronal death. Thus, a vicious cycle becomes operative to cause delayed and progressive neurodegeneration[78] [Figure 3].

Cell autonomous mechanism

Common toxins used in animal PD models such as MPTP, 6-hydroxydopamine, and the aforementioned DSP-4 belong to this class. Initially, these toxins are selectively taken up by neurons and directly cause neuronal damage. Different from the LPS model, these direct-acting toxins usually cause neuronal loss within days without causing acute inflammation during the initial stage. If the damaged neurons are able to secrete enough DAMP to trigger reactive microgliosis, then the vicious cycle will start and drive

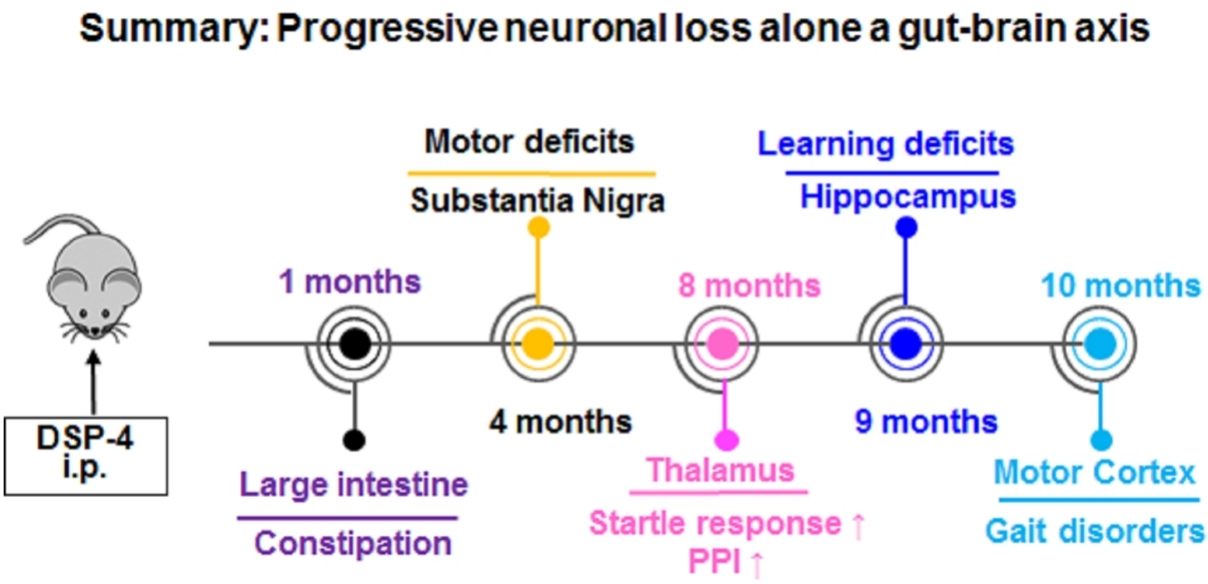

Figure 2. DSP-4 injection causes progressive neuronal loss along the gut-brain axis. DSP-4-induced chronic inflammatory models display progressive ascending neuronal loss along a caudal-rostral axis, which recapitulates the spatiotemporal order of neurodegeneration in PD. Furthermore, the colon is an early site affected after injection with DSP- 4[137]. α-synuclein pathology and enteric neuronal loss were initially found in the large intestine at one month, while neurodegeneration in the brain was observed a few months later, indicating progressive neurodegeneration occurs along the gut-brain axis.

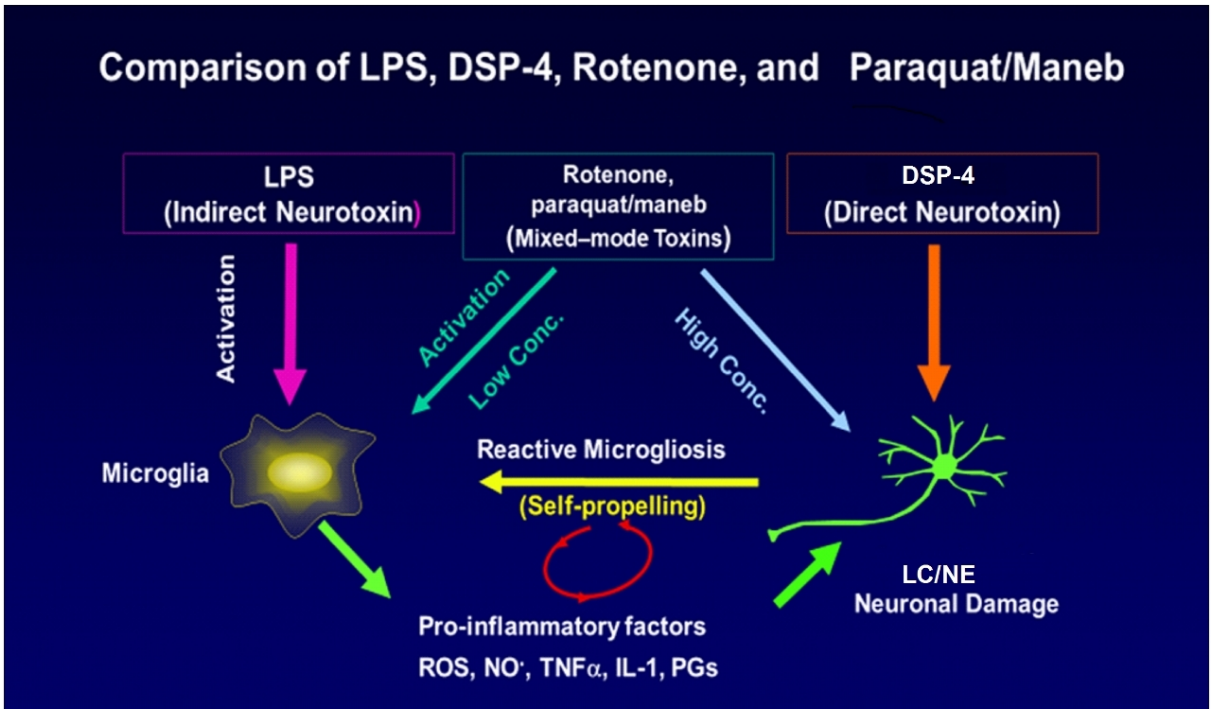

Figure 3. Comparison of LPS, DSP-4, rotenone, and paraquat/maneb. This figure illustrates that toxins produce neurotoxicity through different mechanisms: (1) LPS by activating microglia; (2) DSP-4 by directly damaging LC /NE neurons; and (3) rotenone and paraquat/maneb by exert directing neurotoxicity in high concentrations while in lower concentrations causing activation of microglia. However, between neuronal damage and reactivation of microglia, eventually, these toxins all generate a self-propelling vicious cycle to keep chronic neuroinflammation continued and drive progressive. (This figure was modified from our previous paper with permission[138]).

inflammation-based progressive neurodegeneration[78] [Figure 3].

Mixed-mode mechanism

Many environmental risk factors, such as pesticides, herbicides, fungicides, and heavy metals, display mixed modes of action in causing neurodegeneration. *In vitro* studies revealed that mixed-mode agents may target different cell types depending on toxin concentrations. Rotenone serves as a prototype agent for illustrating this class of toxins. In high concentrations, rotenone can directly damage neurons in neuron-enriched cultures by inhibiting mitochondrial complex I. By contrast, rotenone at low concentrations is not sufficient to directly damage neurons, but it exerts neurotoxicity through microglial activation in neuron-glial cultures[62]. Microglia-dependent neurotoxicity of rotenone has also been reported in an animal study[65] [Figure 3].

Prolonged microglial activation plays a key role in DSP-4-elicited neurotoxicity

A consistent pattern of progressive, ascending, and sequential loss of brain neurons was found in different models of NE-deficient mice, which is similar to that of LPS-treated mice. These findings align with the idea that loss of LC/NE neurons could play a key role in the subsequent neuron loss in other brain regions. To address this question, the time course study of microglial activation after DSP-4 injection was performed. Immunocytochemical analysis using CD11b, a marker for microglial activation, revealed that DSP-4 induced time-delayed microglial activation. Enhanced CD11b-immunoreactivity was not observed until seven days after injection, peaked at two weeks, and remained elevated for up to ten months in NE heavily innervated regions, such as SN, hippocampus, and cortex, but not in the caudate/putamen[36]. PET analysis using [18F]-PBR translocator protein as a ligand for neuroinflammation in DSP-4-injected mice showed similar patterns of increased microglial activation at 10 months after injection[36]. Genetic studies using DBH conditional *KO* mice showed a long-lasting increase in microglial activation compared with *WT* mice (Song *et al*., unpublished observations). Putting all the evidence together, a clear pattern emerges, indicating a high degree of correlation of prolonged microglial activation and neuronal loss in LC/NE-innervated regions in both DBH-genetic knock-out and NE-depleted mice. These findings clearly demonstrate a crucial role of LC/NE in the pathogenesis of PD.

Why is LC/NE particularly vulnerable to the insults of a variety of toxins?

LC/NE neurons are more susceptible to oxidative damage following injections of a variety of toxins/toxicants: LPS, rotenone, paraquat, maneb, *etc*.[79,80]. In PD, the reduced level of NE following LC/NE degeneration is closely correlated with the development of a series of prodromal and nonmotor symptoms[81-84]. It has been reported that depletion of brain NE significantly enhanced neuronal loss in many rodent PD models, including LPS, MPTP, 6-OHDA, and combined paraquat and maneb models[73,85-90]. These findings were further confirmed by our recent studies on both NE-depleted and DBH-deficient conditional knock-out mice[36,72].

To further address the question, we explored the differential vulnerability among various groups of neurons in response to toxic insults. It is generally believed that distinct nuclei respond differently to microenvironments under chronic exposure to oxidative stress and may lead to PD with age[91]. The most vulnerable neuronal populations likely share three intrinsic features: (1) coexist with a large quantity of active microglia[92,93]; (2) impaired antioxidant buffering capabilities[94,95]; and (3) greater energetic demands in neurons with long-axon projections, multi-synaptic neurotransmission, and pacemaker firing[96,97]. In a DSP-4-treated chronic neuroinflammatory mouse PD model, the superoxide/ROS productions were significantly increased in LC and SN in comparison to age-matched vehicle control. However, the appearance of oxidative injuries in the cortex and hippocampus was not observed until a few months later. When antioxidant systems in those nuclei are overwhelmed by too much oxidative stress, it results in the

irreversible dysfunction of mitochondria and cell death[80,98-101]. Thus, the vulnerability to oxidative injuries in different brain regions seems to be the driving force for a discrete, sequential spatiotemporal pattern of neurodegeneration[102]. Indeed, the PET with [18F]-Fluorodeoxyglucose {[18F]-FDG} study clearly showed the high basal levels of glucose consumption in olfactory bulb, thalamus, midbrain, and hindbrain regions in control mice[36,72]. Moreover, the drastic increase in microglial activation, as measured by [18F]-PBR111 uptake, was found in the same brain areas after different toxins challenge[36,72]. Taken together, these results further support the idea that the energy demand and neuronal susceptibility are the key factors that lead to the subsequent oxidative injury-related neurodegeneration in the caudo-rostral order.

Dysfunction of noradrenergic system exacerbates inflammation-based ascending sequential neurodegeneration and behavioral deficits

Besides producing the sequential caudal-rostral pattern of neurodegeneration, noradrenergic dysfunction is associated with both motor and nonmotor behavioral changes in mice. Since the level of NE content reduces with aging, so it is thought to be associated with the appearance of a wide range of nonmotor symptoms as well as contributing to the neurodegenerative process. We hypothesized that selective pre-depletion of NE in an LPS-induced chronic neuroinflammatory mouse PD model may not only accelerate the disease progression but also expedite PD-like nonmotor and motor symptoms. Indeed, we found that mice pre-treated with DSP-4 significantly potentiated LPS-induced neurodegeneration in different brain regions in a sequential, ascending, and time-dependent pattern, such as SN, hippocampus, and motor cortex, but spared in VTA and striatum[72]. Most importantly, aligned with the enhanced neurodegeneration, this "two-hit" model also displayed greater deficits of both nonmotor (e.g., hyposmia, constipation, anxiety, sociability, exaggerated startle response, and impaired learning) and motor (e.g., decreased rotarod activity, grip strength, and gait disturbance) symptoms in a progressive fashion[72]. It is interesting to comment on the clinical relevance of loss of LC/NE neurons in nonmotor dysfunctions of PD patients. The prodromal nonmotor PD symptoms, such as GI disturbance, constipation, orthostatic hypotension, anxiety, and loss of sociability, are likely related to the early loss of LC/NE neurons since adrenergic neurons directly control the autonomic nervous system regulating these functions. Furthermore, the loss of cognition ability in the late stage of PD patients may be related to dysfunction of higher centers, such as the hippocampus and cortex, which are heavily innervated by LC/NE neurons[103,104]. Our DSP-4/LPS mouse PD model recapitulates many nonmotor dysfunctions in a similar temporal fashion[72]. Our mechanistic study demonstrating the relationship among the loss of LC/NE function, chronic neuroinflammation, and neurodegeneration lends strong support for a pivotal role of the LC/NE system in the pathogenesis of PD. Taken together, this novel "two-hit" dosing regimen not only revealed a critical role of early LC lesion in the pathogenesis of PD but also provided an accelerated PD model that recapitulates both PD-like sequential neurodegeneration and progressive appearance of motor/nonmotor symptoms[72].

Molecular mechanism of anti-inflammatory and neuroprotective functions of NE

Besides functioning as a neurotransmitter, NE has also been well-studied in the periphery for its anti-inflammatory capacities[105-108]. We hypothesized a lesion of NE neurons may disrupt brain immune homeostasis results in chronic neuroinflammation and subsequent neurodegeneration. Previous *in vitro* studies demonstrated that NE in micromolar concentrations or higher exert neuroprotective effects[109-111]. Interestingly, a recent report showed that sub-micromolar concentrations of NE (10^{-9}-10^{-6} M) also exert anti-inflammatory and neuroprotective effects in LPS-treated midbrain neuron-glial cultures[73]. The reason for using lower concentrations of NE was that, while micromolar NE can be reached in synaptic junctions[112], sub-micromolar concentrations of NE are probably more relevant for studying its extra-synaptic effects. In the brain, most of NE will be either re-taken up by nerve terminals or undergo enzymatic breakdown. Therefore, it was reasoned that the remaining NE, which escapes from both processes, is capable of acting on the surrounding microglia even at less than micromolar concentrations[113] [Figure 4].

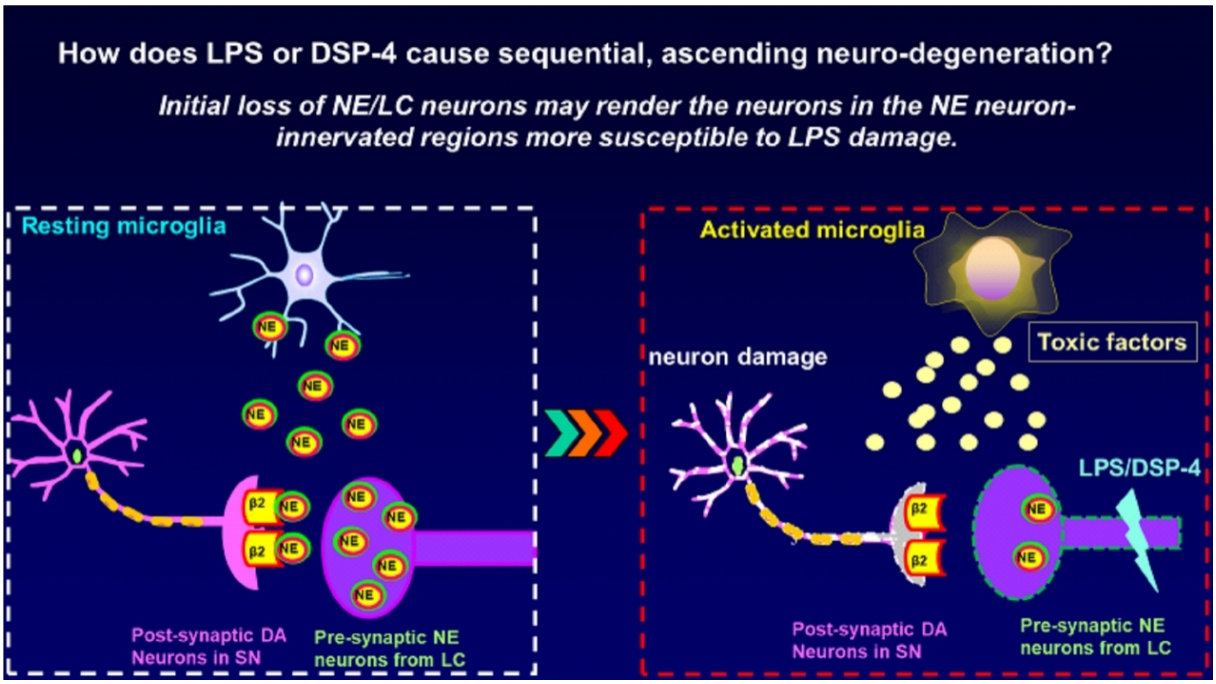

Figure 4. Initial loss of NE/LC neurons resulted from either LPS or DSP-4 injection renders neurons in the NE neuron-innervated regions more susceptible to inflammation-related damage. In normal condition (Left), NE released from the presynaptic terminals of LC/NE neurons performs multiple functions through different ways of transmission. During synaptic transmission, released NE functions as a neuromodulator by directly acting on postsynaptic β2-receptors to modulate the function of postsynaptic neurons. In volume transmission, extra-synaptic NE, diffused out of the synapse or released from dendrites, can act on other neighboring cells, such as microglia. NE exerts anti-inflammatory and neuroprotective functions through the inhibition of microglial NOX2. In pathological condition (Right), reduced NE release from LC/NE neurons not only disrupts the synaptic transmission, but also renders surrounding microglia prone to activation to release proinflammatory immune factors, leading to neuronal damage. Thus, we hypothesize that dysfunction of LC/NE neurons after LPS or DSP-4 injection renders neurons more sensitive to inflammation/oxidative insults and initiates neurodegeneration.

Further studies demonstrated that sub-micromolar NE exerts neuroprotective effects by way of reducing the release of a series of pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNFα) and free radicals (e.g., superoxide/ROS, nitric oxide, *etc.*) from LPS-treated microglia cultures[73].

A novel β2-AR-independent pathway mediating sub-micromolar NE-induced anti-inflammatory effect: inhibition of microglial NOX2-produced superoxide

On immune cells, β2-Adrenergic receptor (β2-AR) plays a critical role in mediating the NE-elicited anti-inflammatory effect by suppressing the release of pro-inflammatory factors via activation of the cAMP/protein kinase A pathway. It is generally accepted that β2-AR mediates the anti-inflammatory effect of micromolar concentrations of NE. It is interesting to find that a novel β2-AR-independent pathway may mediate the sub-micromolar NE-elicited anti-inflammatory effect. We demonstrated that LPS-induced superoxide production was significantly inhibited by NE in a dose-dependent manner in primary midbrain neuron-glial cultures[73]. Two NE optical isomers were used to investigate the important role of ARs in inhibiting NE-derived superoxide production. Surprisingly, the active isomer (-)-NE showed over 100-fold AR-binding affinity than that of inactive isomer (+)-NE[114,115]. However, both (+)-NE and (-)-NE were found equipotent in inhibiting superoxide production in LPS-treated mixed-glia cultures. The AR-independent inhibitory function of both NE isomers on superoxide production was further confirmed in a low AR-expressing cell line, COS7 cells, treated with phorbol myristate acetate (PMA)[116-118]. As expected, after transfected with NOX2, both isomers exerted a comparable inhibitory capacity on PMA-induced

superoxide in COS7 cells[119]. Moreover, the AR-independent inhibitory capacity of both NE isomers has also been confirmed in mouse mixed-glia cultures with genetically depleting ARs[73].

Besides β2-AR, the possibility of the involvement of other types of ARs in regulating NE-elicited reduction of superoxide production has also been studied. However, blocking α1 and/or β1 ARs by pretreating with their non-selective antagonists (phentolamine and propranolol, respectively) failed to show any changes in NE-induced superoxide reduction in LPS-treated mixed-glia cultures[120]. Moreover, inhibition of PKA, a common enzyme in ARs signal transduction pathways, again failed to affect NE-induced superoxide production[73]. Altogether, these findings reveal that NOX2 plays a critical role in regulating sub-micromolar NE-elicited microglial deactivation. It should be emphasized here that β2-AR is still activated by micromolar NE. In fact, our previous report showed that salmeterol, a long-acting β2 adrenergic receptor agonist, exerts a neuroprotective effect against LPS-elicited DA neuron damage mediated through the β2-AR/β-arrestin pathway[120].

NOX2 IS A KEY PLAYER IN DISEASE PROGRESSION AND PRIME TARGET FOR DEVELOPING DISEASE-MODIFYING THERAPY

Recent studies revealed a critical role of microglial NOX2-derived ROS in initiating neuroinflammation-mediated oxidative damage and progressive neurodegeneration[121]. Neuroinflammation has been widely accepted as a crucial contributor to progressive neurodegeneration in a broad spectrum of neurodegenerative diseases[78,122,123]. Microglia can be activated by a wide range of stimuli that are able to disrupt brain homeostasis, such as infection, ischemia, trauma, toxic insults, or autoimmune injury.

Once activated, microglia release innumerable cytotoxic factors, including cytokines, chemokines, proteases, excitatory amino acids, eicosanoids, and ROS. NOX2-derived superoxide has been recognized as one of the most crucial players in chronic progressive neurodegeneration[78,122,123]. Those microglial NOX2-derived ROS (H_2O_2 and peroxynitrite) can directly enter neurons, resulting in impaired mitochondrial integrity, reduced ATP production, and increased mitochondria-derived ROS. They also cause a series of damages to enzymes and other proteins through oxidation, nitration, aggregation, or accumulation (e.g., a-synuclein). By dysfunction of the ubiquitin-proteasome system, ROS will not only reduce protein degradation but also exaggerate abnormal protein accumulation. Moreover, the impaired redox-sensitive signal transduction, products of oxidated DNA, RNA, and lipids, and/or ROS-induced autophagy also play a role in oxidative neuronal damages during neuroinflammation[10,124,125].

Role of dysregulated NOX2 in PD

It has been reported that the increase in microglial NOX2 was found in the SN of both PD patients and mouse PD models[126]. In line with those pathological examinations, a crucial role of microglial NOX2 activation in driving DA neurodegeneration has also been extensively studied[78,127]. For example, in a microglia and DA neuron co-culture system, the mis-folded α-synuclein is able to kill DA neurons by activating microglial NOX2 to release ROS[128]. Moreover, the presence of microglia exacerbates DA neurodegeneration following diverse challenges, including fMLP and LPS, angiotensin II and nanometer-sized diesel exhaust particles, PD-producing neurotoxins (6-OHDA, MPTP, and MPP^+), and PD-associated pesticides (paraquat and rotenone); such neurodegeneration could be alleviated by NOX2 deletion, diphenyleneiodonium (DPI), or apocynin[129]. In addition, the release of DAMPs and other cellular components to the extracellular space, such as high-mobility group box 1, the active form of matrix metalloproteinase-3, or aggregated α-synuclein, could trigger reactive microgliosis and release NOX2-dependent ROS production, which further facilitates DA neurodegeneration[46]. In a MPTP-induced mouse PD model, minocycline-induced neuroprotective effects were achieved by inhibition of microglial activation

and membrane translocation of $p67^{phox}$[130]. Furthermore, the neurotoxic effects induced by either systemic administration of MPTP or intra-nigral injection of LPS were significantly suppressed in NOX2-deficient mice in comparison to *WT* mice[131].

NOX2 is a prime target for anti-inflammatory therapy

Chronic aberrant neuroinflammation, a ubiquitous feature among a variety of neurodegenerative diseases, has been targeted as a disease-modifying strategy for halting the diseases progression[120,132-134]. However, little progress has been made on the ground due to the lack of knowledge pinpointing the immune factors released during chronic neuroinflammation. Recent studies suggest that blocking the superoxide/ROS-generating enzyme NOX2 ameliorates neuroinflammation and reduces neurodegeneration[132].

A NOX2 inhibitor DPI has served as a useful tool to demonstrate the advantages of targeting NOX2 as a prime target for therapy. DPI is a widely used NOX2 inhibitor. However, commonly used concentrations (1-10 μM) of DPI are highly toxic in cell cultures and animals, thus preventing its use in humans. We discovered that an ultra-low dose of DPI (10 ng/kg/day) displayed potent anti-inflammatory and neuroprotective effects in LPS-treated mice[132]. Furthermore, post-treatment of DPI to LPS-treated mice that already shown marked loss of nigral DA neurons and motor symptoms could effectively stop the remaining neuronal population from degeneration and largely restore motor functions[132]. The dopaminergic neuroprotective effects of low-dose DPI, even in a post-treatment regimen, were also detected in an MPTP-induced mouse PD model[132]. Recent studies using similar post-treatment regimens demonstrated the same efficacy of DPI in DSP-4 injected mice[36]. DPI greatly reduced microglial activation, decreased oxidative stress, and most importantly protected DA neurons. Collectively, these findings suggest that DPI is effective at protecting neurons in either infectious agent (LPS)- or non-infectious agent (DSP-4)-induced mouse PD models, suggesting that targeting NOX2 can be a novel and promising therapeutic strategy for PD.

In addition to targeting microglial NOX2, the use of β-AR agonists has also been tried as a potential therapy for PD. It is worth noting that, in LPS-injected mice, post-treatment with the β2 adrenergic receptor (β2-AR) agonist salmeterol significantly rescued DA neurons and improved motor function deficits[120]. Results from these animal studies corroborate a recently published human study. A meta-analysis showed that asthmatic patients prescribed with salbutamol, a β2-AR agonist, had significantly reduced lifetime risk of developing PD[135].

CONCLUSIONS

This review provides clear and convincing evidence to demonstrate that low-grade chronic neuroinflammation is a key factor leading to the progressive neurodegeneration in PD. The reduction of brain NE resulted from the lesion of LC/NE neurons is sufficient to initiate and maintain chronic neuroinflammation, which is associated with progressive, massive, and sequential loss of vulnerable neurons that are sensitive to oxidative damage. Dysregulated microglial NOX2 plays a critical role in generating and maintaining chronic neuroinflammation, oxidative stress, and subsequent neurodegeneration among vulnerable brain regions. NOX2 may serve as a prime target for developing promising disease-modifying therapeutic strategies for PD.

DECLARATIONS

Authors' contributions

Preparing the first draft: Wang QS, Hong JS

Made substantial contributions to conception and revised manuscript: Song S
Give critical comments: Jiang LL

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AUTHOR INSTRUCTIONS

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2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc*.
Here is a guideline of a cover letter for authors' consideration:
In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, *etc*.) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;
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2.2 Types of Manuscripts

There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc*. For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author include an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
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2.3 Manuscript Structure

2.3.1 Front Matter

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The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

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Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

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This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

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Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, Editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

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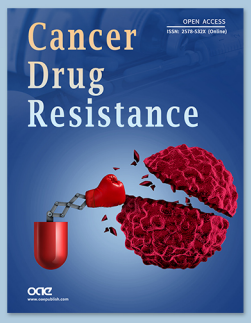

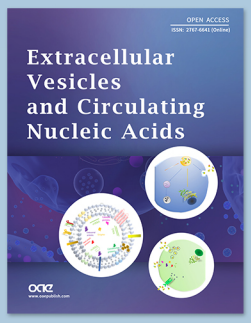

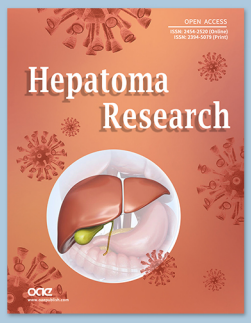

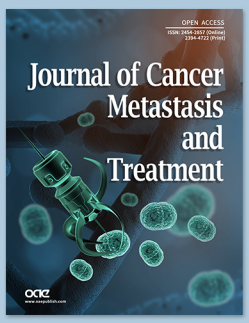

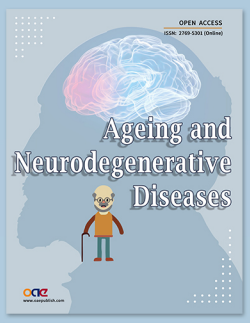

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