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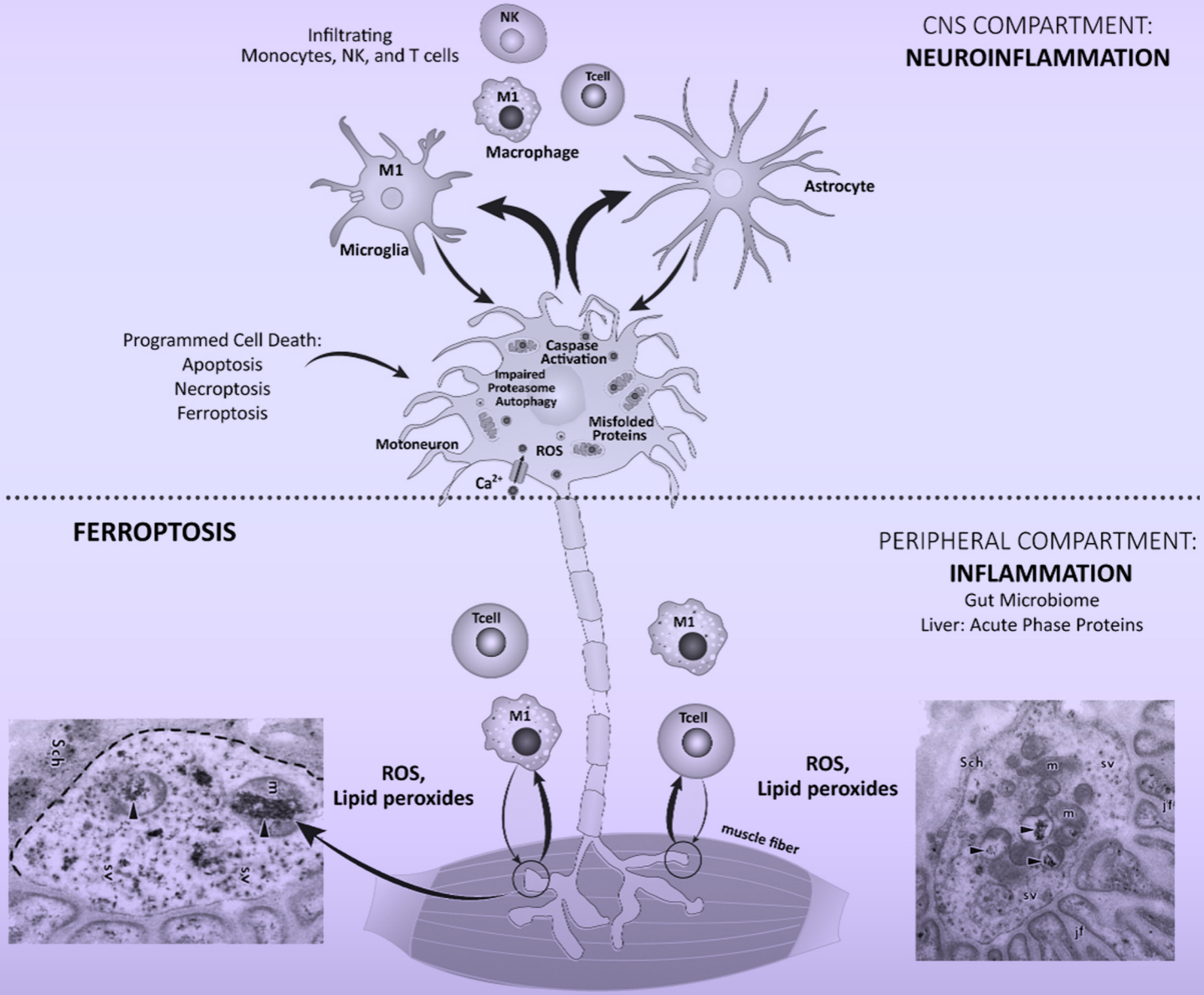
AGEING AND NEURODEGENERATIVE DISEASES



Oxidative stress-mediated inflammation promotes the pathogenesis of amyotrophic lateral sclerosis

Stanley H. Appel

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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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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. Therefore, we plan to launch this new journal, which is aimed 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;
5. Psychology and Geriatric Mental Illness;
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Ageing and Neurodegenerative Diseases

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Ageing and Neurodegenerative Diseases

Perspective

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Ferroptotic cells augment T-cell activation and neuroinflammation

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Abstract

Since ferroptosis, a form of cell death characterized by aberrant lipid peroxidation, was proposed 10 years ago, its interaction with the immune system has been revealed gradually. On the one hand, immune cell-secreted cytokines are able to increase or suppress ferroptosis sensitivities of other cell types, such as tumor cells and fibroblasts. On the other hand, ferroptotic cell-released factors have the capacity to modulate the functions of neighboring immune cells, including dendritic cells, macrophages, and T cells. Identifying these immunomodulatory molecules generated during ferroptosis paves the way for developing novel immunotherapy strategies for treating cancer and autoimmune diseases.

Keywords: Ferroptosis, T-cell activation, experimental autoimmune encephalitis

Ferroptosis is a form of regulated cell death triggered by unrestricted accumulation of lethal lipid peroxides

on cell membranes[1]. Since it was identified 10 years ago, ferroptosis has been shown to be involved in the occurrence or progression of various pathological diseases, including cancer, neurodegeneration, cardiovascular diseases, and acute kidney injury[2,3]. The majority of previous studies have focused on illustrating the cellular intrinsic signaling and metabolic pathways that initiate or prevent the execution of ferroptosis, as well as attempted to clarify the associations between these pathways and cell death-related pathological phenotypes. However, it remains unclear how ferroptotic cells interplay with surrounding cells including immune cells in the pathological tissue microenvironment and whether their interactions contribute to the pathological progression.

Experimental autoimmune encephalitis (EAE) is the most commonly used animal model for multiple sclerosis (MS), which is an autoimmune disease characterized by inflammatory demyelination, oligodendrocyte death, and neuronal degeneration in the central nervous system[4]. MS lesions are mediated by the invasion of immune cells, including $CD4^+$ T cells and monocytes[5]. Although certain features of ferroptosis have been observed in the MS and EAE, including iron overload, reduced expression of glutathione peroxidase-4, and oxidative damage[6], whether ferroptotic cells are really present and involved in the demyelination and MS pathogenesis is still inconclusive. Recently, Luoqian *et al.* observed the accumulations of iron and lipid peroxidation in the cortical tissue of EAE mice and found ferroptosis inhibitor liproxstatin-1 could relieve demyelination and neurodegeneration in animals[7]. As an essential gene for ferroptosis execution, acyl-CoA synthetase long-chain family member 4 (ACSL4) was also found to be increased in $NeuN^+$ neuron cells along the progression of EAE. Knockdown of ACSL4 in the spinal cord reduced lipid peroxidation and ameliorated EAE severity. These results demonstrate that ferroptosis is induced in spinal cords and involved in EAE development.

Myelin autoantigen-specific T cells are major initiators and mediators of MS and EAE, including $CD4^+$ Th1 and Th17 cells[8]. Luoqian *et al.* found that ferroptotic lipid peroxidation was elevated before T-cell activation at the early stage of EAE[7]. Liproxstatin-1 or ACSL4 knockdown could reduce T-cell infiltration and prevent the onset of EAE. To test whether ferroptosis in neuronal cells can directly regulate T-cell function, the supernatant of ferroptotic neurons treated with classical ferroptosis inducer RSL3 or erastin was collected. When naïve $CD4^+$ T cells were activated by anti-CD3 and anti-CD28 antibodies, these ferroptotic supernatants could enhance the secretions of IL-2 and IFNγ from T cells, suggesting that T-cell activation was augmented by certain factors from ferroptotic neurons. Furthermore, the adoptive transfer of T cells that were pretreated by ferroptotic supernatant exacerbated EAE pathogenesis.

Finally, the authors used ceruloplasmin (Cp), a cuproenzyme that can oxidize ferrous iron into ferric iron, to prevent ferroptosis in EAE mice. Cp administration reduced the contents of iron and lipid peroxidases in the spinal cord and decreased the infiltration of $CD4^+$ T cells, resulting in relieved demyelination, neuronal death, and attenuated EAE clinical scores.

Overall, the study by Luoqian *et al.* demonstrated that ACSL4-mediated ferroptosis is induced in neuronal cells during the early stage of EAE progression, and then ferroptotic neurons release certain factors to augment T-cell activation and its effector function, which can accelerate the progression of EAE[7] [Figure 1]. This study is the first to provide evidence that ferroptotic cells play an immunostimulatory role by directly working on T cells, as well as enriching our understanding of how ferroptosis interplays with immune response.

Recently, the interactions between ferroptotic cells and immune cells have been revealed gradually and drawn more and more attention. On the one hand, immune cells are able to modulate ferroptosis sensitivity

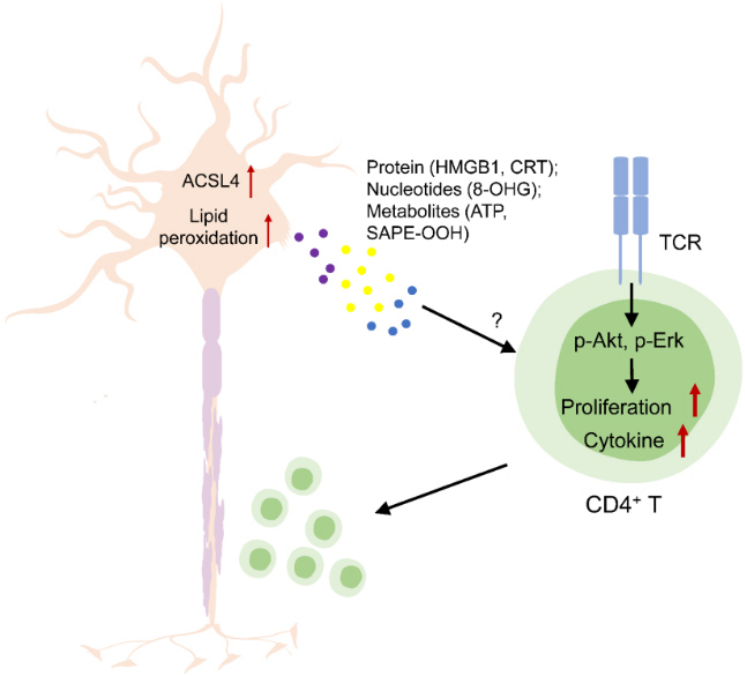

Figure 1. Overview of the mechanism by which ACSL4-mediated neuronal cell ferroptosis augments $CD4^+$ T-cell activation and EAE progression. HMGB1: High-mobility group box 1 protein; CRT: calreticulin; 8-OHG: 8-hydroxy-2′-deoxyguanosine; SAPE-OOH: 1-steaoryl-2-15-HpETE-sn-glycero-3-phosphatidylethanolamine; TCR: T-cell receptor; CD4: cluster of differentiation 4; p-Akt: phosphorylated protein kinase B; p-Erk: phosphorylated extracellular signal-regulated kinase; EAE: experimental autoimmune encephalitis.

of other cells, such as tumor cells. The earliest study revealed that $CD8^+$ T cells activated by cancer immunotherapy could sensitize melanoma cells to ferroptosis through secretion of IFNγ, which suppresses the expression of solute carrier family 7 member 11 (SLC7A11), resulting in limited uptake of cystine by tumor cells[9]. Furthermore, $CD8^+$ T cell-secreted IFNγ was shown to coordinate with arachidonic acid to directly induce tumoral ferroptosis in the absence of synthetic molecules[10]. Combinations of checkpoint blockade and ferroptosis activators, such as an enzyme degrading cystine and cysteine or arachidonic acid supplementation, have synergistic antitumor activities across multiple murine tumor models[9,10]. In contrast to the ferroptosis sensitization effect of IFNγ, some inflammatory cytokines can prevent ferroptosis. Interleukin-6 was shown to inhibit ferroptosis of head and neck squamous cell carcinoma cells by JAK2/STAT3-mediated upregulation of SLC7A11[11]. Tumor necrosis factor (TNF), another T cell-secreted cytokine, was able to protect synovial fibroblasts from ferroptosis by increasing system xc^- expression and cystine uptake. In the collagen-induced arthritis mouse model, a TNF blockade combined with a ferroptosis inducer synergistically initiated ferroptosis in synovial fibroblasts and attenuated arthritis progression[12]. Therefore, in different inflammatory scenarios, cytokines secreted by immune cells regulate the ferroptosis of their neighboring cells distinctly by reprogramming the metabolisms of fatty acids or amino acids.

On the other hand, ferroptotic cells can be sensed and processed by immune cells, including macrophages or dendritic cells, to modulate innate and adaptive immune responses. Ferroptosis was initially considered an immunogenic cell death (ICD), a type of cell demise that can elicit uptake of cellular components by dendritic cells (DCs) and enhance antigen presentation to T cells, resulting in the activation of antigen-specific cytotoxic T-cell response. The earliest evidence shows that ferroptotic cells could release damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1) and calreticulin (CRT) exposure[13-15], which could all function as immune adjuvants to promote the activation

and maturation of DCs[16]. Later, Efimova *et al.* reported that the early ferroptotic MCA205 cells induced by short-term treatment of RSL3 stimulated maturation of bone marrow-derived DCs and induced a vaccination-like effect *in vivo* in contrast to late ferroptotic cells[17]. Another recent study also used the same MCA205 cells whose ferroptosis was induced by ML162 or GPX4 knockdown; however, it drew the opposite conclusion that ferroptotic cells were not immunogenic regardless of the stage of cell death, even though they could release ATP, HMGB1, and cytokines including CXCL1 and IFNβ. Mechanistically, engulfed ferroptotic cells suppressed the expressions of pro-inflammatory genes and impaired antigen cross-presentation in DCs[18]. The above results suggest that ferroptotic tumor cells caused by different inducers may have different immunomodulatory effects due to certain unique molecules released by these cells. One of the crucial features of ferroptosis is the accumulation of lipid peroxides such as oxidized phospholipids of plasma membranes. Although an oxidized phospholipid [1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC)] was shown to impair the differentiation and immune-stimulatory function of *in vitro* cultured DCs[19,20], it is unknown whether this oxidized PAPC is enriched in ferropototic cells. A recent study identified another oxidized phospholipid, 1-steaoryl-2-15-HpETE-sn-glycero-3-phosphatidylethanolamine (SAPE-OOH), which is generated during ferroptosis of leukemic cells and functions as an eat-me signal to promote phagocytosis of ferroptotic cells by macrophage[21]. In addition to oxidized lipids, oxidized nucleobases, such as 8-hydroxy-2'-deoxyguanosine (8-OHG), were also found to be released by GPX4 deficient pancreatic cancer cells. 8-OHG induced macrophage infiltration and activation via TMEM173, resulting in immunosuppression and tumor progression[22]. Therefore, although it is still controversial whether ferroptosis is immunogenic or not, specific molecules generated and released from ferroptotic cells would modulate the functions of DCs or macrophages and the subsequent engagement of T-cell response.

Furthermore, it would be worth knowing whether ferroptotic cells have direct impacts on other immune cells, especially T cells. Luoqian and colleagues provided the first evidence that some factors released from ferroptotic cells work on T cells directly to enhance their activation and effector function. These mysterious factors are present in the conditioned medium from ferroptotic primary neurons treated with RSL3 or erastin. Although the identities of these factors are not revealed yet, they have the ability to amplify the signaling transduction of T-cell receptors, including activations of Akt and Erk. These data inspire our interest in further investigating the characteristics and identities of these T cell-promoting factors, although it will be more rigorous to test the effects of trace residuals of RSL3 or erastin in the supernatant from ferroptotic cells on T cells.

Altogether, ferroptotic cells could be immunosuppressive or immunostimulatory due to their broader impacts on various types of immune cells, including DCs, macrophages, and T cells. Identifying these immunomodulatory molecules generated from ferroptotic cells will be the top priority of future research in this field. It is also worth knowing whether these ferroptosis-related immunomodulatory molecules are cell type-specific. In other words, can the same factor act on multiple types of immune cells? A further question is: Do different ferroptotic cells release different sets of immunomodulatory molecules? For example, other than neurons, can other types of ferroptotic cells release the same T cell-promoting factors? The answers to these questions hold promise for developing novel therapeutic approaches to treat cancer or autoimmune diseases.

DECLARATIONS

Authors' contributions

Conceptualization, writing-review & editing: Lu F, Wang W

Writing-original draft: Xue Y
All authors read and approved the final manuscript.

Availability of data and materials
All data described in the article are provided within the article.

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Conflicts of interest
All authors declared that there are conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
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Ageing and Neurodegenerative Diseases

Perspective

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Genome-edited rabbit, a prospective alternative model for neurological diseases

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Abstract

Animal models have great importance in the research of human neurodegenerative diseases due to their value in symptom mimicking, mechanism investigation, and preclinical tests. Although non-human primate and large animal models have good performance in disease modeling due to their high maintenance cost and critical ethical standards, rodent models are commonly used. Rodent models have been successfully applied in modeling many neurological diseases; however, their genetic background, neuroanatomical features, and nervous system development are different from those of humans. Moreover, the short lifespan and small body size of rodent models also limit the monitoring of disease progression and observation of clinical symptoms in studying neuronal disorders that are late-onset or have a long course of progression. In comparison with rodents, rabbits are phylogenetically closer to humans and have closer similarities to humans in brain development, thus are an alternate animal model for human neurological diseases.

Keywords: Neurodegenerative diseases, genome editing, animal model, rabbit, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis

INTRODUCTION

Neurodegenerative diseases (NDDs) are associated with progressive neuron losses, most of which are linked with genetic disorders[1]. At present, neurological disorders are considered as one of the major causes of mortality and disability worldwide[2]. Unfortunately, many NDDs are late-onset and hard to detect in the early stage; for instance, Parkinson's disease (PD) and Alzheimer's disease (AD) have high morbidity in elderly patients, while amyotrophic lateral sclerosis (ALS) does not exhibit clear symptoms at the early stage of the disease. Moreover, due to the irreversibility of neuronal death, no effective therapeutic approaches are available at present. Therefore, a thorough investigation of these diseases is essential for the development of disease-specific and effective prognostic, diagnostic, and therapeutic strategies. Model organisms are essential platforms for the above research; cell lines and animal models are frequently used. Although cell models can be used for the investigation of pathological pathways and molecular mechanisms of disease pathogenesis[3], due to the limitation in modeling organogenesis and human physiology[4], they cannot mimic histological, morphological, and behavioral changes in human diseases. Therefore, animal disease models that partially recapitulate the aspects of human diseases are essential. Additionally, animal models have irreplaceable value in preclinical tests; they are also important for the development of prognostic and therapeutic strategies.

In comparison with non-mammalian animals such as zebrafish and *Drosophila melanogaster*, mammalian models have greater similarities to humans in genetics, metabolism, and physiology and can therefore mimic some of the biological and clinical features of human disease[5,6]. In practical research, large animal models are often used because of the biological characteristics of these animals for modeling human NDDs[7]. With the development of genome editing tools and somatic cell nuclear transfer (SCNT) techniques, genetically modified large animal models can be effectively produced, which can promote the utilization of these models[8]. Unfortunately, maintenance costs, space requirements, and ethical standards are still problems for the use of large animal models in biomedical research. Compared with large animal models, rodents have a small body size, low maintenance cost, and can be easily handled, making them cost-efficient models. Moreover, rodents have a relatively high genetic identity and physiological similarity to humans, and genetic modification capabilities can facilitate the modeling of genetic disorders[9]. Therefore, currently, rodents are the main experimental animal for biomedical research and disease modeling. From 1950 to 2010, approximately 80% of animal-based biomedical studies were performed on rodents (59% on mice and 18% on rats)[10].

Rodents have been applied in modeling many neurological diseases and have adequate precision in mimicking the pathology and physiology in some cases. However, due to factors such as lifespan, genetic differences from humans, and small body size, rodent models have some limitations in studying neuronal disorders[9]. For example, they cannot replicate the exact pathological hallmarks in some human diseases because of physiological and genetic differences. PD mouse models (α-synuclein transgene or knockout of *LRRK2*, *PRKN*, and *PINK1*) do not show degeneration of nigrostriatal dopaminergic neurons[11] and striatal neurons remain viable, which is different from the pathological features in human disease[11]. The absence of intranuclear inclusion body formation in neuronal cells of ALS mice overexpressing mutant *hSODl* is inconsistent with the phenotype of human ALS disease. Thus, finding an alternative animal species for modeling is needed to produce better models for diseases that cannot be recapitulated in mice.

Rabbits are docile and easy to handle; their short reproductive cycle and high reproductive performance can guarantee an abundant sample size for experiments; and the efficiency of model production and the low demand for rearing and surgical operation equipment make rabbits easy to maintain and handle[12]. Moreover, rabbits have an intermediate lifespan (longer than rodents but shorter than large animals such as

non-human primates), and, compared with rodents, rabbits are phylogenetically closer to humans[13] and are more similar to humans in brain development[14]; therefore, they may have better precision in disease modeling. With the development of targeted genome editing tools, producing targeted genome-edited rabbit models for human neuronal disorders has become attainable.

ADVANTAGES OF RABBITS AS ANIMAL MODELS OF NEUROLOGICAL DISEASES

Rabbits are phylogenetically closer to humans than mice

Genetic similarity to humans is linked with the identity of protein structure and function, and high genetic similarity could increase the precision of disease modeling. Compared with rodents, rabbits are phylogenetically closer to primates[13], suggesting they may have better precision in disease modeling. Notably, some human genes do not have orthologs in mice: approximately 1% of human genes cannot find orthologs in mice's genomes. For instance, caspase 10, a gene that is linked to neurodegeneration via the extrinsic apoptosis pathway[15], is absent in mice but has orthologs in rabbits[11]. However, the phylogenetic similarity between rabbits and humans does not guarantee rabbits would be a better model for all human diseases; the performance of disease modeling is still dependent on the type of mutant gene, and the mechanism involved in the pathogenesis should also be considered.

The development of the central nervous system of rabbits has greater similarity to humans compared with rodents

Neurological features are critical for NDD modeling, and the CNS development of rabbits is highly similar to that of humans compared with that of rodents. Specifically, the phase of brain development and myelination in rabbits is more similar to humans than that of rodents, since such a process happens during the perinatal period in humans and rabbits but postnatally in rodents[14]. Moreover, rabbits have a higher brain volume and cerebral surface area than mice. The time point of morphological configuration of major CNS structures of rabbits is closer to humans. The development of structures such as primitive streak, neural tube closure, and primary brain vesicles in rabbits is chronologically similar to that in humans[14]. In addition, the development of white matter in rabbits is closer to that of humans[14], and rabbits have a higher white matter ratio than mice (approximately 20% *vs.* 10%)[16,17] [Table 1]. Moreover, rabbits have larger brain volume, cortex surface area, and number of neurons compared with rodents [Table 1], suggesting that rabbits may exhibit better cognitive, learning, and memory abilities.

Rabbits can be trained to learn basic skills (e.g., recall signals) through positive reinforcement[25]. Rabbits also have both short- and long-term memory[26,27] and can exhibit memory losses when mimicking NNDs such as AD[27]. Specifically, in an AD rabbit model constructed by drug induction, the results of novel object recognition (NOR) and object location memory (OLM) tests suggest that the model can track cognitive impairment[28]. In other studies, the results of conditional and unconditional response tests also suggest that the AD rabbit model has reduced learning ability[29,30].

Additionally, axon degeneration is a common pathological feature of NDDs, and neurons with longer projections have a higher vulnerability to axon degeneration, which can be easily affected in NDDs[31]. Some mice models of motor neuron diseases exhibit molecular pathological features in neurons but only exhibit mild or even no behavioral symptoms[32,33]; vulnerability to axon degeneration might be the explanation for this phenomenon, since the axon length in rodents is shorter than that in larger animals. Collectively, rabbit models might have better accuracy in mimicking human neuronal diseases compared to rodents.

Rabbits have a relatively large body size for handling and sampling

In addition to the genetic and neurological features, rabbits also have a bigger body size compared with rodents, which can facilitate better animal handling and symptom observation. The relatively large body size

Table 1. Major differences in size and structures between rabbits and mice

	Human	Rabbit	Mice
Brain volume[18]	1300-1400 g	10-13 g	0.4-0.5 g
Spinal cord length[18]	43 to 45 cm	18 cm	7.5
Gray-white matter ratio[16,17]	40:60	80:20	90:10
Duration to reach adult brain volume[19,20]	20 years	4 months	2 months
Glia-neuron ratio (GNR)[21]	1.66	0.32-0.49	0.29-0.42
Number of neurons[22-24]	86,000 million	494.2 million	71 million

of the rabbits is also associated with larger organ size and blood volume (45-75 mL per kg body weight versus 1.5-2.5 mL)[10,34], which can be beneficial for diagnostic investment, surgical operation, and sampling for pathological analysis. Cerebrospinal fluid (CSF) and blood biomarker analyses are commonly used in the diagnosis of NDDs such as AD and FTD[35-37]. Such assays are hard to perform in mice due to the poor sample size, especially for experiments that need continuous monitoring; in contrast, for larger animals such as rabbits, an adequate amount of sample can be collected with minimal harm to the animal.

In addition, medical imaging approaches are usually needed for the prognosis and diagnosis of NDDs[38,39]. However, it is hard to perform high-definition medical imaging on small animals due to the limitation of the equipment. High-resolution magnetic resonance imaging (MRI) in mice requires a scanner with an ultra-high field strength of 7 T or higher[40], which is inaccessible for most researchers. Compared with rodents, rabbits have larger CNS [Table 1]; thus, a normal MRI scanner with a 3.0 T field strength is adequate for CNS imaging in rabbits[41]. The large body size of rabbits can also benefit electromyography tests, which are commonly used in the diagnosis of neuromuscular diseases such as ALS. Additionally, the scale of the central neuron system also affects the maneuverability of tissue sampling and the intraparenchymal or epidural injection of therapeutic vectors such as AAV in future translational medical research.

Collectively, the larger body size of rabbits makes it easier to handle and sample compared with small animals, which largely facilitate phenotype observation and surgical operation.

The lifespan of rabbits is long enough for the observation of disease progression

The onset of neurodegenerative disease and the speed of progression are affected by both genetic and environmental factors[42]. For pathological mutations that induce late onset and slow progression, the lifespan of animal models should also be considered, since the effect of aging can interfere with the observation of clinical symptoms[9]. Generally, NDDs are progressive diseases that last from years to decades. For instance, the median survival time of ALS patients is 20-48 months[43], while that of AD patients can reach up to 30 years[44]. Moreover, late-onset NDDs such as AD and PD develop late in life; both diseases usually begin at age 60 or older in human patients[45]. However, the normal lifespan of mice is 12-36 months[10], which means that for diseases that begin late or have a slow rate of progression, mice may not fully exhibit the whole course of the disease. For mutations that can only induce late-onset symptoms or slow progressive disease, mice models may not exhibit observable symptoms in their lifetime without extra administration, such as drug stimulation[46]. Furthermore, due to the short lifespan of mice, it is hard to identify whether a symptom (e.g., vision loss) is caused by pathological neuronal death or age-related reasons[9]. In the adult phase, 2.6 mice days is equivalent to one human year, while, in the post-senescence phase, 2.069 mice days is equivalent to one human year[10]. Such fast senescence processes can largely limit the progression of disease and interfere with the observation of disease-related clinical symptoms. In contrast, the maximum lifespan of laboratory rabbits can reach up to 10 years under proper conditions, and

one human year is equivalent to 18.25 and 50.34 rabbit days in the adult and post-senescence phases, respectively[34]. Thus, for most NDDs, the lifespan of rabbits is long enough for the observation of disease progression.

PRODUCTION OF GENOME-MODIFIED RABBIT DISEASE MODELS VIA CRISPR-CAS SYSTEM

The production of disease models that recapitulate the pathological features of human disease is an important approach to investigating the pathogenesis of the disease. Artificially induced disease models can exhibit clinical features of some NDDs. For instance, hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, rotenone, and paraquat are commonly used in the induction of Parkinson's disease[47]. However, many NDDs are caused by pathological mutation of the disease-related gene, and an induction model cannot fully recapitulate the whole pathological pathway of diseases caused by genetic disorders[1]. Therefore, to elucidate the whole pathogenesis process of neuron degeneration, the production of animal models that carry pathological mutations that mimic human disease is necessary.

With the development of gene-editing tools, efficient and accurate genome modification has become achievable. To date, various genome-edited rabbits have been constructed, as shown in Table 2. In 2013, the CRISPR-Cas9 system was harnessed for efficient targeted genome editing in eukaryotic cells[99,100]. Moreover, with the further development of research on CRISPR-Cas systems, the CRISPR-Cas systems and their derivates can facilitate targeted gene knockout (KO), knockin (KI), activation, suppression, and single-base substitution. Presently, various genome editing tools based on CRISPR-Cas systems are widely used in multiple species, including non-human primates, large non-primate animals, rodents, and rabbits[101-103].

The first CRISPR-Cas-mediated gene KO in rabbits was successfully generated in 2014[101] [Table 2]; however, full-length gene KO can only recapitulate diseases caused by loss of function. To mimic diseases caused by gain-of-function mutation due to point mutation, more accurate gene manipulation is needed. Furthermore, more than 50,000 disease-causing mutations in humans are point mutations; therefore, a novel system that can mediate single base substitution is needed. Since 2017, the development of cytosine and adenine base editing systems can facilitate efficient C to T and A to G base substitutions, which can facilitate precise gene manipulation[104]. Such systems were identified as having ideal editing efficiency in rabbits [Table 2]; the efficiency of cytidine base editor (CBE) and adenine base editor (ABE) in rabbits after co-microinjection of base editor mRNA and sgRNA are 53%-88% and 44%-100%, respectively[90]. The following refinement of base editors has overcome or reduced the limitations of PAM sequences and the incidence of bystander activities[92,105]. At this stage, base editing systems are capable of inducing disease causative missense and nonsense mutations in rabbits to generate disease models.

Although base editing systems can induce four transversion mutations, it is impossible for such systems to induce the other eight transversion mutations. Moreover, the generation of bystander mutations cannot be completely avoided when there are multiple C or A in the editing window. Importantly, conventional gene editing systems cannot induce efficient single base or oligonucleotide insertions and deletions. Therefore, it is hard to generate disease models with fragment shift mutations. Fortunately, the development of prime editing systems solved such problems in 2019. The system, which is based on the target binding capacity of the CRISPR-Cas9 system and the retro-transcription activity of retrotrancripsase, can facilitate the whole genome "search and replace" activity in organisms. Prime editor was successfully used in generating a Tay-Sachs disease (TSD) rabbit model in 2021 [Table 2], which is a model of neurological disease generated by prime editor-mediated four base insertion[98].

Table 2. Summary of genetically modified rabbits

System	Genes	Modification	Application	Refs.
ZFN	*IgM*	KO	Immunodeficiency	[48]
ZFN	*APOC3*	KO	Lipid metabolism and atherosclerosis	[49]
ZFN	*APOE*	KO	Lipid metabolism and atherosclerosis	[50]
ZFN	*CETP*	KO	Lipid metabolism and atherosclerosis	[51]
TALENs	*RAG1; RAG2*	KO	Immunodeficiency	[52]
TALENs	*FAH*	KO	Hereditary tyrosinemia type 1	[53]
CRISPR/Cas9	*FBN1*	KO	Marfanoid progeroid lipodystrophy syndrome	[54]
CRISPR/Cas9	*DMD*	KO	Duchenne muscular dystrophy	[55]
CRISPR/Cas9	*ANO5*	KO	Muscular dystrophy	[56]
CRISPR/Cas9	*α-Crystallin*	KO	Congenital cataracts	[57]
CRISPR/Cas9	*GJA8*	KO	Congenital cataracts	[58]
CRISPR/Cas9	*LDLR*	KO	Lipid metabolism and atherosclerosis	[59]
CRISPR/Cas9	*MSTN*	KO	Muscle hypertrophy	[60,61]
CRISPR/Cas9	*SRY*	KO	Sex reversal syndromes and hermaphroditism syndromes	[53,54]
CRISPR/Cas9	*PHEX*	KO	X-linked hypophosphatemia	[62]
CRISPR/Cas9	*LMNA*	KO	Premature aging syndrome	[63]
CRISPR/Cpf1	*WRN*	KO	Werner syndrome	[64]
CRISPR/Cas9	*TYR*	KO	Oculocutaneous albinism	[65,66]
CRISPR/Cas9	*DMP1*	KO	Mineralization defects	[67]
CRISPR/Cas9	*GADD45G*	KO	Congenital cleft palate	[68]
CRISPR/Cas9	*HOXC13*	KO	Hair and nail ectodermal dysplasia	[69]
CRISPR/Cas9	*GCK*	KO	Maturity-onset diabetes of the young type 2	[70]
CRISPR/Cas9	*HBB2*	KO	β-thalassemia	[71]
CRISPR/Cas9	*WAS*	KO	Wiskott-Aldrich syndrome	[72]
CRISPR/Cas9	*CBS*	KO	Congenital hyper-homocysteinemia	[73]
CRISPR/Cas9	*LDLR; APOE*	KO	Lipid metabolism and atherosclerosis	[74]
CRISPR/Cas9	*APOC3*	KO	Lipid metabolism and atherosclerosis	[75]
CRISPR/Cas9	*CFTR*	KO	Cystic fibrosis	[76]
CRISPR/Cas9	*CFTR*	KO ΔF508	Cystic fibrosis	[77]
CRISPR/Cas9	*CLPG*	KO	Muscular hypertrophy syndrome	[69]
CRISPR/Cas9	*FGF5*	KO	Long hair	[78]
CRISPR/Cas9	*IL2RG*	KO	X-linked severe combined immunodeficiency	[79]
CRISPR/Cas9	*MC1R*	KO	Block the synthesis of eumelanin and create a novel pale-yellow coat color	[80]
CRISPR/Cas9	*XIST P1*	KO	X-chromosome inactivation	[73]
CRISPR/Cas9	*MSTN*	KO	Muscle hypertrophy	[74]
CRISPR/Cas9	*PCSK9*	p.S386A	Lipid metabolism and atherosclerosis	[75]
CRISPR/Cas9	*ATP7B*	p. R778L	Wilson Disease	[81]
CRISPR/Cas9	*TYR*	p. T373K	Oculocutaneous albinism	[82]
CRISPR/Cas9	*TYR*	KO	Oculocutaneous albinism	[83]
CRISPR/Cas9	*RAG; RAG2; TIKI1; ALB; IL2RG*	Multiplex gene KO	Immunodeficiency	[84-86]
CRISPR/Cas9	*FUT1; FUT2; SEC1*	KO	Fucosyltransferases enzymes activity	[87]

CRISPR/Cas9	*ROSA 26*	KI	Safe harbor gene	[88,89]
BE3	*MSTN*	p.Q93stop	Muscle hypertrophy	[90]
	TYR	p.Q68stop	Oculocutaneous albinism	
ABE7.10	*DMD*	p.T279A	Duchenne muscular dystrophy	
eAID-BE4max	*TYR*	p.R299H	Oculocutaneous albinism	[91]
YFE-BE4max	*TYR*	p.Q68Stop	Oculocutaneous albinism	[92]
nNme2-CBE	*FGF5*	p.Q79Stop	Long hair	[93]
eA3G-BE	*TYR*	p.Q48stop	Oculocutaneous albinism	[94]
NG-ABEmax	*HOXC13*	p.Q271R	Hair and nail ectodermal dysplasia	[95]
BE4max	*FGF5*	Start Codon Disruption	Long hair	[96]
ABE8.17	*TYR*	p.T325A	Oculocutaneous albinism	[97]
	LMNA	p.L530P	Emery-Dreifuss muscular dystrophy	
PE3	*HEXA*	p.Y427fs	Tay-Sachs disease	[98]

PROSPECTS AND LIMITATIONS FOR EVALUATING RABBIT DISEASE MODELS

The observation of the clinical phenotypes of diseases is important for the evaluation of animal models. However, unlike the well-developed testing platforms for rodent models, currently, the evaluation criteria for rabbit NDD models are not well established.

In general, the diagnosis, prognosis, and autopsy criteria in human NDDs can be used in animal models. Such investigations can provide data that are comparable to human clinical reports and have better referential value. Indeed, commercialized analysis platforms, such as serological testing, enzyme-linked immunosorbent assay, MRI, electromyography, and histological analysis, are versatile and authentic tools for the assessment of both humans and animals including rabbits. However, it is impossible to apply the whole set of human diagnostic criteria to animals. For example, the investigation methods for behavioral and cognitive analyses in humans are hard to apply in animal models. Standardized and species-specific behavioral analysis platforms can support the assessment of animal disease models. For rodents, systematic behavioral analysis systems are well established and standardized; systems such as multivariate concentric square field and cylinder test are used to investigate traits such as sensory-motor function[106]. In contrast, the behavioral and cognitive analysis platforms for rabbits are not well developed at present, and further development of these systems is necessary for the future use of rabbits in neurological disease modeling.

CONCLUSION

Collectively, rabbits are more similar to humans in brain development, with more genetic similarities than rodents, and longer lifespan and larger body size, suggesting that rabbits can perform well in human neurological disease modeling in addition to traditional non-human primates, large animals, and rodent models. Therefore, it is expected that, in the near future, with the further development of genome editing technology and the establishment of phenotype assessment platforms for rabbit models, the value of rabbits in the research of neurological diseases can be maximized, not only for the understanding of pathological mechanisms but also for innovation of therapeutic approaches.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Zhang Z, Song Y

Performed data acquisition, as well as provided administrative, technical, and material support: Li Z, Lai L

Availability of data and materials
Not applicable.

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All authors 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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Rat models of major neurodegenerative disorders

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Abstract

No single animal model can recapitulate all the features of a particular human disease on its own. Historically, rats have been used to study neurobiology and underlying functional networks. Likewise, rat models have been created to study neurodegenerative mechanisms and therapeutic interventions. In the last decades, a shift towards the use of mice has been observed in many research fields, not least because of the comparatively easier genetic manipulation of mice. However, with the full sequence of the rat genome being available, advances in genetic manipulation of the rat, and advanced test regimens and biomarkers at hand, the rat presents itself once more as a valuable model organism for studying neurodegenerative disorders. This review provides an overview of currently available, well-characterized rat models of Alzheimer's disease, Parkinson's disease, and Huntington's disease, as well as their advantages for studying neurodegenerative disorders and evaluating therapeutic interventions.

Keywords: Genetic rat models, phenotypic rat models, Alzheimer's disease, Parkinson's disease, Huntington's disease

INTRODUCTION

Rattus norvegicus, the laboratory rat, was the first mammal to be domesticated and kept in captivity for research purposes[1,2]. Over time many inbred rat strains have been obtained to study various physiological aspects, disease mechanisms, and pharmacological questions. Both mice and rat models have been relied on

in basic and preclinical research. Their short generation times, relatively easy to establish housing conditions, and genetic similarities to humans have made them the largest group amongst animal models in research (EU commission, 2019 report). In general, rats are considered an ideal species for behavioral studies, and have been used far more than mice in behavioral research in the past, although the increasing use of transgenic mice in behavioral testing in recent years has inverted this trend[3]. Rats are easy to handle by experimenters and less aggressive towards conspecifics (i.e., members of the same species) than mice[4]. Rat behavior has been well characterized, and several behavioral tasks currently used in rodents may better fit the rat[5,6], as they were originally developed in rats[7,8]. In cognitive tests which are used to model cognitive deficits of human disorders, especially for tasks requiring swimming, such as the Morris water maze, rats display less floating and thigmotaxis[9] and perform better than mice[10,11], probably because they are adapted to the water environment and are natural swimmers. In a decision-making task, rats were shown to learn the task faster than mice[12]. Furthermore, compared to mice, rats display a more complex behavioral repertoire which is likely to result from the species' evolutionary history[13]. Increasing evidence in the last 15 years suggests that, similar to primates, rats present metacognition, that is, the awareness of one's own cognitive processes[14-16]. In the context of neurodegenerative disorders (NDs), this is relevant given that metacognitive impairment is a feature of Alzheimer's disease (AD) and other dementias[17].

Consequently, using rats to model cognitive symptoms could increase the robustness of cognitive assessments and enhance the accuracy of phenotypes. However, it is important to bear in mind that different rodent species differ in their behavioral traits[18,19] that could best mimic specific aspects of a human disorder, emphasizing the importance of using multiple model species, especially given the heterogeneity of deficits in several neurodegenerative disorders.

The rat's body size further offers advantages over mice and other small animal models, as surgical procedures can be performed more reliably and consistently. Repeated blood and cerebrospinal fluid (CSF) sampling of larger volumes is possible in rats, and neuroimaging and electrophysiological measurements are preferentially performed in rats. The rat remains the classical animal model in toxicological studies, as the eradication of toxins is more closely related between human and rat, than between human and mouse[20]. However, a close examination of the individual biological processes affected is necessary, as many differences exist between species[21]. Both mouse and rat genomes were published in the early 2000s[22,23] opening the way for genetic studies investigating rat genes that share similar traits in rats and humans[20]. With the advancement of genetic tools, mice have been favored over rats due to technical challenges in creating rat models carrying genetic mutations. By improving methods for harnessing rat embryonic stem cells and advances in genetic tools, like zinc finger nuclease and CRISPR/Cas systems, rat models have been created more successfully in the last two decades. However, with a certain time delay in comparison to respective mouse models[24].

For NDs, like AD, Parkinson's disease (PD), and Huntington's disease (HD), no natural mutation in the rat exists that would provide a rat strain to model the human disease. Therefore, rat lines have been created that mostly carry and overexpress the human disease gene, in order to elicit phenotypes that resemble pathology and behavioral alterations, reminiscent of what is observed in patients. However, the most prevalent NDs, AD and PD, are not monogenetic disorders, with a low proportion of familial cases and, therefore, inherently difficult to model.

AD is the most common neurodegenerative disorder. Patients suffer from progressive cognitive decline, affecting, for example, memory and orientation and with disease progression limiting activities of daily life. The decline in cognitive abilities and behavioral alterations are caused by preceding, exaggerated amyloid

beta (Aβ) peptide plaque formation and tau tangles. Progressive neuronal loss in the hippocampus and other brain regions further leads to reduced levels of neurotransmitters[25].

PD, like AD, is a highly prevalent neurodegenerative disorder that has a multifactorial etiology and is most often of idiopathic origin. Genetic and environmental factors contribute to the disorder that is primarily characterized by the lack of the neurotransmitter dopamine, leading to bradykinesia and other motor deficits in patients. Several PD-causing and PD-risk genes have been identified. Mutations in α-Synuclein (*SNCA*), Parkin (*PARK2*), PTEN-induced kinase 1 (*PINK1*), Protein deglycase DJ-1 (*DJ-1*), and Leucine-rich repeat kinase 2 (*LRRK2*) amongst others can cause the familial form of the disorder. On the cellular level, PD is characterized by mitochondrial dysfunction, altered protein degradation pathways, and increased neuroinflammation leading to synaptic dysfunction and neuronal loss in the substantia nigra pars compacta[26].

HD is a monogenetic ND caused by a CAG repeat expansion in exon 1 of the *huntingtin* gene (*HTT*), which translates to a poly-glutamine tract in the huntingtin protein (HTT)[27,28]. HD commonly manifests in adulthood, with CAG expansions in a range of 36 to 60 CAG repeats[29]. More than 60 CAG repeats are associated with juvenile HD, leading to symptom onset before the age of 20 years[30]. The neuropathological hallmarks of HD are extensive cell loss in the striatum and HTT aggregates localized in the neuropil, perikarya, and nucleus[31-33]. The clinical manifestations include motor deficits, cognitive impairment, and psychiatric disturbances[34].

This review provides an overview of rat models that have been generated to study the above-mentioned NDs, AD, PD, and HD. Neuropathological characteristics and behavioral phenotypes of well-characterized genetic models are summarized and stand in contrast to phenotypic/aspect-replicating rat models that are historically and currently more commonly used in biomedical research. We aim to highlight the advantages both types of rat models offer in terms of readouts and study design opportunities to improve translatability to human treatment.

GENETIC RAT MODELS TO STUDY AD, PD, AND HD

Neuropathological phenotypes

Neurodegenerative diseases represent a large group of neurological disorders with progressive loss of particular subsets of neurons. The most common NDs are Alzheimer's disease (AD) and Parkinson's disease (PD); and as a monogenic disease, Huntington's disease (HD), is well-studied. In addition to the progressive and selective neuronal cell loss, the second central characteristic of NDs is the presence of protein aggregates composed of misfolded proteins, specifically, the N-terminal fragment of mutant huntingtin in HD, Aβ peptide and hyperphosphorylated tau in AD, and α-synuclein (α-syn) in PD. The role of protein aggregates in NDs, whether neurotoxic or neuroprotective, is still a matter of debate since the distribution of protein aggregates does not reliably match the patterns of neuronal loss in different diseases[35]. Nevertheless, due to its commonality among NDs and its dependency on a specific molecular cascade (i.e., misfolding, oligomerization, and fibrillization), protein aggregate formation remains an important aspect of ND research. Thus, animal models that recapitulate the disease's characteristic protein aggregation pathologies can make great contributions to understanding the disease mechanisms and aid in the development of therapeutic strategies. For genetically modified animal models of NDs, the presence, as well as the regional and subcellular location of protein aggregates, depends on the genetic construct's promoter, protein expression levels, and genetic background of the animal. Mouse models have closely recapitulated the features of human NDs and provided essential insight into neuropathology. However, no single animal model can mimic all aspects of human diseases, not even all mouse models, collectively. Rats

and mice are closely related species, but still have genetic and physiological differences, such as the distinct expression pattern and localization of certain protein isoforms. These diversities lead to some variance between both species in resembling human pathological processes, making rat models a meaningful complement to mouse models. This section discusses the commonly used genetic rat models for AD, PD, and HD [Table 1], and describes to what extent they recapitulate the characteristic protein aggregate pathology.

Neuropathological phenotypes in genetic rat models of Alzheimer's disease: APP^{NL-G-F} knock-in, TgF344-AD and McGill-R-Thy1-APP transgenic rats

Amyloid plaques containing Aβ peptide and neurofibrillary tangles (NFTs) consisting of hyperphosphorylated microtubule-associated protein (tau) make up the typical protein aggregate forms in AD. While some studies suggested that tangles may precede plaques, it is commonly accepted that the amyloid plaques are formed first and trigger tau agglomeration (see review[49]). Nevertheless, both amyloid plaque and tau tangles are characteristic features of AD. The development of amyloid plaques appears to be dependent on the initial accumulation of Aβ, which is derived from amyloid beta precursor protein (APP) through sequential proteolytic cleavage by β and γ-secretase. Mutations in APP close to the main APP cleavage site and in the catalytic subunit of γ-secretase presenilin (*PSEN*) are major genetic causes of familial AD[50,51]. Ultimately, overexpression of APP with a combination of multiple mutations has been used to generate *APP* transgenic models[52-54], while double transgenic models expressing mutant *APP* and mutant *PSEN* represent *APP/PSEN* models (see review[36]).

Many transgenic *APP* mouse models recapitulate amyloid plaque formation and disease manifestation of AD and have thereby made essential contributions to understanding Aβ pathology in familial AD. In comparison, *APP* rat models often develop less accumulation of Aβ peptide and amyloid plaques. This cannot be simply explained by lower expression levels of transgenes in rats, or different transgene protein isoforms, or the applied promoters. One rat model, however, displays full amyloid pathology. Leon *et al.* developed an *APP* transgenic rat model expressing *hAPP751* under the control of the murine Thy1.2 promoter and containing the Swedish and Indiana mutations of APP (McGill-R-Thy1-APP rats)[55]. This rat model carries one copy of the transgene hemizygously and accordingly presents approximately double the amount of APP protein (i.e., both endogenous and transgenic) as wild-type rats. Homozygous rats show an early-onset and progressive accumulation of Aβ peptide starting at 1 week of age and develop extracellular Aβ deposition at 6 months of age. Particularly, at 20 months of age, McGill-R-Thy1-APP transgenic rats display dense-core plaques in most brain areas with predominant presence in the entorhinal and parietal cortices, and hippocampus, the typical brain structures that are vulnerable to AD[56-58]. In summary, despite the lower expression level of the transgene, McGill-R-Thy1-APP transgenic rats develop early-onset, progressive, characteristic amyloid plaque pathology making this model valuable for studying Aβ pathogenesis in a close to physiological condition.

In fact, the distribution and burden of amyloid plaques in AD patients do not correlate with neuronal loss, disease severity, or disease duration. In contrast, NFT formation strongly correlates with neuronal death and follows a typical progression from the frontal cortex and the CA1 area of hippocampus to the anterodorsal thalamus, and in later stages (IV), the CA4 region of hippocampus[59,60]. Instead, NFTs have only been found in AD mouse models carrying human mutant tau, mostly with P301L mutation[36]. P301L missense mutation in tau is the genetic cause of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17); this mutation causes tau hyperphosphorylation and its subsequent aggregation into NFTs[61,62]. Different from FTDP-17, tau is not the only hyperphosphorylated neuronal protein in AD, and hyperphosphorylated tau is the result of a protein phosphorylation/dephosphorylation unbalance (see

Table 1. Genetic rat models of Alzheimer's, Parkinson's, and Huntington's disease

	FAD		FPD			HD	
Categories (Example)	Early onset AD (McGill-R-Thy1-APP rats, TgF344-AD)	Later onset AD (APOE epsilon 4 knock-in)	Autosomal recessive (PINK1 KO, DJ-1 KO)	Autosomal dominant (α-synuclein BAC, LRRK2 KO)		Juvenile-onset HD (BACHD)	Adult-onset HD (tgHD)
Molecular and biological basis	Mutation in APP, PSEN1, hyperphosphorylation of tau	rRsk factors, e.g., APOE variants	Mainly loss-of-function, e.g., PARKIN, PINK1 and DJ-1	Mainly gain-of function, e.g., SNCA, LRRK2		CAG expansion > 60	CAG expansion < 60
Strategy	• Expression/knock-in of a combination of multiple mutations in APP • Expression of mutations in APP + PSEN • Expression of mutations in MAPT	• Humanization of loci of AD relevant mutations	• Knock out of PARKIN, PINK1 or DJ-1	• Overexpression of wild-type or mutant SNCA	• Overexpression of wild-type or mutant LRRK2 • KO	• Overexpression of full-length mutant HTT	• Overexpression of the N-terminal fragment of mutant HTT
Pros	• Early and progressive recapitulation of neuropathological features • Tangle-like pathology • Spatial cognition deficits	• Physiological levels of protein expression	• Mitochondrial pathology can be studied • Cranial sensorimotor deficits can be studied (DJ-1 KO and PINK1 KO)	• LB pathology can be studied	• Effect of impaired dopamine homeostasis can be studied	• Early and progressive recapitulation of the HTT aggregation phenotype • Displays HD-like behavioral phenotypes	• Represents the major form of HD • Displays HD-like behavioral phenotypes
Cons	• Non-physiological condition • Represents a small portion of disease form (early onset AD accounts for < 5% of AD cases)	• Need to verify and characterize identified novel risk factors	• Most of them do not mimic the LB pathology of PD patients? • No motor behavior impairment in PARKIN KO		• No dopaminergic neurodegeneration	• Represent only juvenile HD • Long CAG repeats may change the HTT protein properties	• Mild and slow recapitulation of disease pathology
Literature	Reviewed in[36-39]	Reviewed in[40-42]	Reviewed in[43-47]			Reviewed in[48]	

AD: Alzheimer's disease; APOE: apolipoprotein E; APP: amyloid precursor protein; CAG: polyglutamine; DJ-1 (PARK7): Parkinson's disease protein 7; FAD: familial Alzheimer's disease; FPD: familial Parkinson's disease; HD: Huntington's disease; HTT: huntingtin; KO: Knockout; LB: Lewy body; LERRK2: leucine-rich repeat kinase 2; MAPT: microtubule-associated protein Tau; PINK1: PTEN-induced kinase 1; PSEN: presenilin; SNCA: synuclein alpha.

review[63]). This raises the debate of whether the P301L resulting tau aggregation can represent tau pathology in AD. Tau is a microtubule-associated protein stabilizing microtubules in the polymerized state[64,65]. Alternative splicing of tau in humans generates six isoforms containing microtubule-binding domain, including three (3R) or four (4R) microtubule-binding repeats[66]. It has been shown that rats express all six tau isoforms as humans, while mice only possess 3R tau isoforms[67].

The TgF344-AD rat is an AD transgenic model that carries transgenic constructs, expressing both the Swedish human mutant *APP* and the *PSEN1*(*PS1ΔE9*). These rats exhibit 2.6-fold human APP and 6.2-fold human presenilin-1 expression, respectively, compared to the endogenous rat homologs. Around 16 months of age, TgF344-AD rats develop amyloid plaques, some of which are thioflavin S-positive dense-core plaques. Strikingly, abundant insoluble tau structures have also been demonstrated in the cortex and hippocampus of aged transgenic animals, whose morphology recapitulates human NFTs. Frank and progressive neurodegeneration combined with neuroinflammation and cell apoptosis have been found in the same brain areas[68]. Similarly, tangle-like tau aggregates were also observed in a wild-type rat injection

model expressing mutant *APP* and *PSIN1*($PS1_{M146L}$) mediated by adeno-associated viruses[69].

Very recently, Pang and colleagues generated an *APP* KI rat model, App^{NL-G-F} rats, which carry three family *App* mutations G676R, F681Y, and R684H[70]. Both homo- and heterozygous rats manifested amyloid plaques rapidly at 1 and 4 months of age, respectively. Notably, the amyloid plaque manifestation in App^{NL-G-F} rats preceded faster in females compared to males[71]. Whether this sex difference in Aβ aggregation can be linked to the higher incidence rates of AD in women than in men requires further investigation. Interestingly, aggregated tau was found in 12-month-old homozygous App^{NL-G-F} rats and further manifested into NFTs at 22 months of age. Increased gliosis, apoptotic cell death and brain atrophy have been observed in App^{NL-G-F} rats at 12 months of age and older.

Taken together, several *APP* rat models have shown common AD neuropathological features in AD-affected brain areas, in particular NFT formation, a key pathogenic event in the disease process, which have not been recapitulated in *APP* mouse models. The lack of the 4R isoforms in mice may be the cause for the two rodents' differing abilities to model human tau pathology.

Neuropathological phenotypes in genetic rat models of Parkinson's disease: PINK1 KO, DJ-1 KO, and a-synuclein BAC rats

The characteristic neuropathological features of PD are intracellular α-synuclein positive inclusions known as Lewy bodies (LBs), and selective neuronal loss in the substantia nigra, which is strongly related to mitochondrial dysfunction (see review[72]). About 20 genes have been identified to cause familial PD, inherited in an autosomal dominant or recessive mode. In the following, we will focus on three PD genetic rat models, which made significant contributions to the PD field as compensations for mouse models: the α-synuclein transgenic rats, *PINK1 KO* rats, and *DJ-1*-KO rats.

α-synuclein BAC transgenic rat model

The major component of LBs is α-synuclein, which is encoded by the *SNCA* gene. This was the first gene revealed to have a causal link to PD development. To this date, six autosomal dominant *SNCA* point mutations (A53T, A30P, E46K, G51D, H50Q, and A53E) have been identified[73]. Moreover, duplication, triplication and quadruplication of the *SNCA* locus have been reported to be causal in genetically unrelated PD families[74-77]. A number of transgenic mice models bearing human mutant or wild-type *SNCA* have been generated. Many of these models exhibit proteinase K resistant, detergent-insoluble, and thioflavin S positive α-synuclein aggregates (see review[78]). Mouse models also show a neuronal loss in PD-relevant brain areas, that is, substantia nigra, neocortex, and hippocampus[79-83]. An α-synuclein BAC transgenic rat model using a bacterial artificial chromosome (BAC) construct consisting of full-length human wild-type *SNCA* locus with the upstream regulatory promoter sequences has been generated by the Riess lab[84]. These BAC transgenic rats showed key pathological features of PD, including progressive misfolding and accumulation of α-synuclein aggregates, striatal dopamine depletion, decreased TH-positive cell numbers, and characteristic dark dopamine neurons in the substantia nigra. These pathological features have been modeled comparably in α-synuclein transgenic mice. However, with larger body sizes, rats offer unique possibilities for surgical manipulations of the brain, serial sampling of cerebrospinal fluid and blood, and brain imaging.

Rat models for autosomal recessive mutations

Autosomal recessive forms of PD commonly present an early onset phenotype[85,86]. All three known autosomal recessive PD genes, *PARKIN*, *PINK1*, and *DJ-1*, are closely associated with mitochondrial dysfunction[87-89]. The PTEN-induced kinase 1 (PINK1) and Parkin are involved in the same pathway leading

to the degradation of damaged mitochondria. PINK1 acts as a sensor for depolarization of mitochondrial membrane potential[85,90-93], recruiting the E3 ubiquitin ligase Parkin which ubiquitinates substrates on the outer mitochondrial membrane, thus eliciting a vicious cycle resulting in mitophagy[94]. Protein deglycase DJ-1 is a stress-dependent chaperone localized in mitochondria, which plays an essential role in ATP production and complex I activity[95,96]. Interestingly, it has been observed that Lewy bodies can be absent in PD patients with either *PARKIN*, *PINK1*, or *DJ-1* mutation (see review[97]). In comparison, mitochondrial pathology and neuronal loss in animal models of autosomal recessive PD are expected as important pathological phenotypes. *PINK1* knockout (KO) rats show decreased complex I level and increased proton leak in the electron transport chain, indicating a mitochondrial respiration defect, as well as a reduced number of TH-positive neurons and proteinase K resistant α-synuclein aggregates[47,98]. By contrast, no evidence reflecting neurodegeneration was found in *PINK1* KO mice[99], not even in the triple knockout mice with deficiency of Parkin/PINK1/DJ-1, all known gene deficiencies related to autosomal recessive PD forms[100]. Similarly, *DJ-1* KO rats show significantly progressive neuronal loss with approximately 50% dopaminergic cell loss at 8 months of age in the substantia nigra, combined with altered mitochondrial respiration[101,102]. In contrast to rat models, no dopaminergic neuron loss-related event or mitochondrial dysfunction has been observed in all existing *DJ-1* KO mouse models, while one *DJ-1* KO mouse model only shows increased sensitivity to the neurotoxin MPTP[103-105]. Notably, *PARKIN* KO mice and rats also have been generated, while *PARKIN* KO mice exhibited increased striatal extracellular DA concentration, which is opposite as expected[106], *PARKIN* KO rats did not show any neuropathological differences compared to wild-type controls[101]. Whether these results can be explained by the genetic and biological differences between human and rodent remains unaddressed. Nevertheless, both *PINK1* and *DJ-1* monogenic KO rat models are valuable for investigating mitochondrial pathology in autosomal recessive PD, whereas the comparable mouse models lack disease-related neuropathological phenotypes.

Neuropathological phenotypes in genetic rat models of Huntington's disease: tgHD and BACHD rats

To date, two genetic rat models have been generated and well characterized for HD research. One carries the whole genomic sequence and regulatory elements of human *HTT* with 97 mixed CAG-CAA repeats in a bacterial artificial chromosome construct (BACHD rats), thereby bearing the mutation in its appropriate genomic context as in HD patients[107]. The interruption in CAG repeats avoids somatic instability of polyQ size and variation in repeat length within the animal colony. The other rat model carries N-terminal rat *Htt* cDNA fragments under the rat *Htt* promoter, with 51 CAG repeats (tgHD rats)[108]. In humans, the CAG length present in the tgHD construct would lead to an adult-onset of disease, whereas 97 CAGs, as in the BACHD rats, would result in the juvenile form of the disorder. Both BACHD and tgHD rat models have a wide expression pattern of transgene *HTT*/*Htt* throughout the brain that, to some extent, resembles the human condition. BACHD rats have a 4.5-fold higher expression level of transgenic *HTT* as the endogenous *Htt*, while tgHD rats show a strongly reduced transgene expression level compared to the endogene[107,108]. Both rat models show subtle evidence for neurodegeneration, including structural changes in white matter[109,110], reduced brain volume in BACHD rats[111], and age-dependent enlarged ventricles in tgHD rats.

Although neuronal loss in HD patients is most prominent in the striatum, mHTT aggregates have been more frequently detected in the cerebral cortex. Subcellular localization studies revealed a prevalent neuropil localization of mHTT aggregates, while smaller amounts of mHTT inclusion bodies were found in the nucleus[31-33]. One of these studies reported that in all 12 investigated HD brains, only 1%-4 % of striatal neurons had nuclear inclusion bodies, while a large number of mHTT aggregates were detected in the cortex with prominent subcellular localization in neuropil and perikarya. Although juvenile HD patients show an increased number of nuclear inclusion bodies compared to patients with adult-onset, neuropil aggregates were still predominantly distributed in the striatum and cortex[32]. Consistent with these

observations, BACHD rats exhibit more prominent mHTT aggregates in the cerebral cortex compared to subcortical areas, with aggregates distributed through all cortical layers, primarily in neurites. tgHD rats display a similar aggregate distribution pattern. Notably, tgHD rats display abundant mHTT aggregates in the dorsomedial part of the striatum and BACHD rats have been found to show a similar aggregate load in the lateral striatum. Interestingly, both HD rat models show a prevalent distribution of HTT aggregates in the limbic structures, with notable aggregate loads in the ventral striatum (nucleus accumbens), striatal terminal bed nucleus, and central nucleus amygdala[107,112,113]. In the BACHD rats, aggregates were also found in the hippocampus and hypothalamus. It is difficult to judge to what extent this relates to human disease, as the distribution of aggregates outside the striatum and cortex has barely been studied in HD patients.

In contrast to the aggregate pathology seen in patients and rat models, most mouse models display nuclear inclusion bodies rather than neuropil aggregates. Moreover, they display more abundant aggregates in the striatum compared to the cerebral cortex, regardless of the genetic construct or modification they are based on[114]. It is therefore clear that BACHD and tgHD rats provide a meaningful complement to HD mouse models for modeling and understanding the mHTT neuropathogenic mechanisms. mHTT aggregation is affected by several intrinsic factors, including polyQ-flanking sequences of mHTT, mHTT interaction partners, protein fragmentation, and post-translational modifications (see review[115]). Different subcellular localization of aggregates may initiate different cellular quality-control processes, resulting in different pathogenic processes. Working with a combination of mouse and rat models of HD, could therefore help tease apart what exactly causes one type of pathology over the other.

Behavioral phenotypes

Behavioral phenotypes in genetic rat models of Alzheimer's disease: APP^{NL-G-F} knock-in, TgF344-AD, and McGill-R-Thy1-APP transgenic rats

Memory impairment is an early symptom in AD patients, followed by language and mathematical deficits, decreased visuospatial orientation, and attention deficits[116,117]. One of the most common symptoms in subjects affected by AD is an impairment of spatial navigation which is the ability to define and retain trajectories between places[118]. Although attributing cognitive functions to specific brain areas does not embrace the complexity of brain networks regulating cognition, hippocampus and medial entorhinal cortex represent essential areas for spatial navigation[119] and are already affected in the early phases of AD[120]. Similar brain areas in humans and rodents appear to be involved in the regulation of specific types of memory, for example, spatial memory[9,121,122], which is important for modeling cognitive deficits in animal models.

Most of the behavioral results in AD genetic models come from the characterization of mouse models. On the other hand, the use of genetic rat models is increasing, and these models may be advantageous from a behavioral perspective, given that cognitive testing is central to AD research. In McGill-R-Thy1-APP transgenic rats, spatial learning and memory deficits already manifest by 3 months of age, prior to amyloid plaque deposition and are present in both homozygous and hemizygous rats which can sometimes differ in the degree of impairment. Spatial cognition deficits include reference and working memory impairment as detected in maze tasks for spatial learning, and problems with object location memory[55,123-125]. TgF344-AD transgenic rats show spatial cognition deficits as early as 4 months of age[126,127]. Similar to the McGill-R-Thy1-APP rats, they were shown to have a deficient performance in several paradigms for spatial cognition including tasks for reference and working memory[68,126,128,129] as well as reversal learning[68,130,131]. Moreover, TgF344-AD rats display a decreased accuracy in spatial trajectories[132]. In line with the results in the transgenic models of AD, five months old APP^{NL-G-F} knock-in rats were reported to display impaired spatial learning abilities[70]. Hence, defective spatial cognition is reproduced among different categories of AD genetic rat models.

A crucial factor in the process of translating behavioral readouts from animal models to humans is the similarity of the deficits measured in each species. Using similar assessments in animal models and patients is of great advantage, as this could ultimately increase the predictability of therapy effects. Accordingly, hippocampus-dependent navigation tasks, commonly used in rats, for example, the Morris water maze, were adapted for humans in the form of real and virtual versions, and revealed impairments in spatial memory and navigation abilities in AD subjects[133,134], consistent with results in transgenic rat models assessed in mazes for spatial learning[55,123,125,132]. Comparative water maze testing in healthy humans and wild-type rats showed a similar effect of scopolamine and donepezil normally used to model cognitive dysfunction and to treat cognitive deficits, respectively[135], indicating similar behavioral responses to pharmacological cholinergic modulation across species. The direct comparison of AD patients and genetic AD rat models would be more informative regarding the analogy between human and rat results in the context of AD.

Episodic memory, which allows to store and retrieve information about personal experiences along with the related spatial and temporal contexts, is dysfunctional in AD[136]. Recognition memory and associative learning, linked to episodic memory, are impaired as well[137-139]. McGill-R-Thy1-APP and TgF344-AD rats display deficits in some aspects of recognition memory and associative learning. In both rat models, deficits in novel object recognition have been reported, although results are overall mixed[123,124,140-143]. There are also signs of associative learning impairment in passive avoidance setups[142,144,145]. Additionally, fear conditioning analyses revealed that multiple memory recall components are impaired in homozygous and hemizygous McGill-R-Thy1-APP rats[124]. Moreover, testing on automated touch screen setups showed impaired associative learning in the McGill-R-Thy1-APP rat model and deficits in episodic-like memory in APP^{NL-G-F} knock-in rats[70,146]. Touchscreen methods like those applied in McGill-R-Thy1-APP rats are meaningful as analogous to platforms applied to assess cognition in AD patients[147].

A large portion of AD patients suffers from subtle neuropsychiatric symptoms, and the most common are apathy, depression, anxiety, and sleep disturbances[148]. Neuropsychiatric disorders, especially depression, have been associated with phenomena such as decreased hippocampal volume, inflammation, and alterations of the monoaminergic systems[149-152]. Mood alterations in rodent models of AD and other neurodegenerative disorders are most commonly assessed in terms of anxiety and depression-like behavior. Both phenotypes have been more extensively characterized in the TgF344-AD rat model relative to the McGill-R-Thy1-APP model. In TgF344-AD transgenic rats, anxiety-like behavior was detected at different ages in the elevated plus maze[128,145,153,154]. In McGill-R-Thy1-APP rats by the age of 5 months, there is evidence for anxiety-like behavior in the light-dark box[125]. Results obtained in the open field in both rat models are contradictory[123,125,143-145,154]. Regarding depression-related parameters, anhedonia-like behavior as well as behavioral despair were shown in TgF344-AD rats aged 10 months or older[131,145,154]. One of these studies assessed both males and females but did not report sex differences[131]. Nevertheless, given the evidence for sex differences in the prevalence of depression and apathy in AD[155], it would be worth examining sex differences more thoroughly in transgenic rat models. Also, the time course of depression-like phenotypes and cognitive impairment in TgF344-AD rats cannot be easily defined from the behavioral analyses in the model. Moreover, given that in AD, depression can predate cognitive symptoms[156], the assessment of depression-like behaviors in animal models from very early ages would be advisable. Apathy, the most frequent behavioral disturbance in AD[149], has not been assessed in detail in the genetic rat models reviewed here. Signs of apathy-related behavior could be inferred from the presence of anhedonia-like behavior and the reduced motivation to engage in goal-directed behaviors in some experiments in TgF344-AD; for example, rats display a decreased number of attempts in a maze test[128]. Similarly, in mouse models of AD, parameters of object and social exploration, as well as locomotor activity, have been used as

measures of apathy[157,158]. Alternative approaches, e.g., progressive ratio tasks[159], used in AD mice[160], may provide more compelling information on apathy-related motivational aspects.

Sleep disturbances are tightly linked to mood and behavioral disturbances. Sleep behavior characterization in 17-month-old TgF344-AD rats showed changes in sleep architecture, such as increased sleep fragmentation and alterations in sleep microstructure, consistent with the sleep alterations observed in the prodromal phase of AD[161]. Sleep analyses in McGill-R-Thy1-APP rats are lacking, although changes in circadian activity have been reported in this rat model by the age of 8-10 months[125]. In conclusion, both the McGill-R-Thy1-APP and TgF344-AD rat models reproduce the dysfunction in key memory aspects, typical of AD patients. Similar deficits are found in APP^{NL-G-F} knock-in rats, although only limited information is available on their phenotype so far, as this is a recent model. Neuropsychiatric changes have been examined in more detail in the TgF344-AD rats which manifest anxiety- and depression-like behaviors as well as sleep disruption. Apathy, a key symptom of AD, remains instead largely unexplored in these models.

Behavioral phenotypes in genetic rat models of Parkinson's disease: PINK1 KO, DJ-1 KO, and a-synuclein BAC rats

Typical motor symptoms in PD patients are bradykinesia, impaired fine motor skills, tremor, muscle rigidity, and deficits in gait, posture, and balance[162-164]. Homozygous *PINK1* KO and *DJ-1* KO rats display numerous abnormalities reminiscent of the human PD symptomatology. They have deficits in limb motor coordination and balance as well as rearing, gait and grip strength[46,101,165-168]. *DJ-1* KO rats additionally show postural instability[167], whereas *PINK1* KO rats display decreased locomotor activity[101,165]. Interestingly, female *PINK1* KO rats do not exhibit limb motor deficits like the ones observed in males of comparable age[169], indicating possible sex differences in the sensorimotor phenotype or in the age when the phenotype becomes manifest. Similar to the other models, the main features of motor impairments in α-synuclein BAC rats are decreased activity and rearing, impaired balance, and gait deficits, although most motor abnormalities in these rats start later compared to *PINK1* KO and *DJ-1 KO* rats[84,170,171]. Tremor, present in PD patients, was, to the best of our knowledge, not reported in the literature for any of these models. Fine paw skills for which specific assays are established in rodents[172,173] have been scarcely assessed, despite the impairments of fine motor skills and hand grasping in PD patients[162,164].

Olfactory dysfunction, dysphagia (i.e., difficulty swallowing), as well as hypokinetic dysarthria, a speech motor control disorder involving reduced voice loudness and altered articulation, are important components of PD symptomatology in a high percentage of patients[174,175]. These changes are not responsive to standard dopaminergic treatments[176], and knowledge of the underlying brain changes is rather limited.

Altered phonation in PD patients has been related to the rigidity of the phonatory posture of the larynx, and laryngeal muscle impairment has been associated with deficient motor control by the basal ganglia[174]. Moreover, an altered perception of speech volume in PD patients[177] has been suggested to result in poor control of speech production[174]. Studies in PD patients also showed deficits in the production and perception of speech-related emotions. The latter seems to be connected with cognitive impairment in the disease[177].

Vocalization in humans and ultrasonic vocalizations in rats share similar anatomical structures and neural pathways[178-182]. The periaqueductal gray, especially, plays an important role in the control of vocalization in mammals[183]. It receives motor and sensory inputs[183] as well as input from multiple limbic areas including cortex, amygdala, and hypothalamus[184-186] that could regulate social and motivational aspects of vocalization. The periaqueductal gray has also been linked to vocalization deficits in PD. This is consistent

with several results in animal models: (i) in mice overexpressing α-synuclein, vocalization deficits are paralleled by α-synuclein aggregates in the periaqueductal gray[187]; and (ii) in *PINK1* KO rats, gene expression analyses identified associations between the expression of specific gene modules in this brain region and female vocal behavior[188].

Both the *PINK1* KO and *DJ-1* KO rat models exhibit ultrasonic vocalization deficits[46,47]. *DJ-1* KO rats display an altered call profile and produce ultrasonic vocalization with decreased intensity, as reported between 2 and 8 months of age[46]. Similarly, male and female *PINK1* KO rats have a decreased ultrasonic vocalization average intensity at the same age[47,169], although opposite observations have been reported regarding ultrasonic vocalization intensity in male *PINK1* KO rats at a later age[189]. The vocalization intensity deficits in *PINK1* KO rats are stronger compared to *PINK1* KO mice[190]. The decreased ultrasonic vocalization intensity in genetic rat models resembles the decreased vocal intensity or loudness in PD subjects, which occurs in the early disease stages. Given that the vocalizations recorded in male and female rats are experimentally induced by exposure to a female and male, respectively, it remains unclear whether a possibly altered interest in the conspecific of different sex may have contributed to this phenotype in *PINK1* KO rats. This is important for two reasons: (i) decreased sexual interest and sexual dysfunction are reported in PD patients[191], and (ii) brain areas controlling vocalization in rats are also involved in sexual behavior[192,193]. Moreover, the connection of the periaqueductal gray, controlling rat vocalization, with limbic areas may involve emotional and cognitive aspects in control and in the impairment of vocalization, which would be interesting to assess in rat models of PD.

Characterization of vocalizations in *PINK1* KO male rats indicated progressively decreased peak frequency[189] and altered bandwidth[47] of frequency-modulated calls, in addition to deficits in call intensity. Although translating these changes from rats to patients seems not as straightforward as the vocalization intensity, the examined variables may be relevant indicators of vocalization dysfunction in rat models. Besides altered vocalization, similar to PD patients, both *PINK1* KO and *DJ-1* KO rats present early oromotor abnormalities[46,47,194]. Already at early ages, *DJ-1* KO rats have a decreased ability to regulate tongue force[46] and *PINK1* KO rats display an altered tongue function and biting deficits[47]. Videofluoroscopy, normally used to detect swallowing deficits in PD patients[195], showed that *PINK1* KO rats are dysphagic as assessed at the age of 4 months[194]. Hence, *PINK1* KO and *DJ-1* KO rats seem promising models regarding phenotypes of cranial sensorimotor dysfunction. However, the information on olfactory abilities in these rat models remains scarce. Sixteen-month-old DJ-1 rats were shown to have increased olfactory abilities, which is opposite to observations in patients[167]. On the contrary, analyses in the BAC α-synuclein rats detected smell discrimination impairment at 3 months, before the appearance of motor deficits[84], which would temporally mimic the manifestation of symptoms in human PD.

PD patients show non-motor symptoms, including psychiatric and cognitive symptoms, sleep disorders, and autonomic dysfunction[196-199]. Most PD patients experience disturbances such as apathy, anxiety, depression, and psychosis and several studies on PD have also reported disorders of impulsive control[197]. Even though some disturbances, for example, psychosis and impulsive control, may in part arise from or be enhanced by treatments, neuropsychiatric symptoms are already observed in the early phases of the disease[197,198,200]. Despite the obvious limitations in translating neuropsychiatric assessments between animal models and humans, genetic rodent models still offer the possibility to relate neuropsychiatric-like behaviors to relevant brain changes on multiple levels in treatment-free conditions, and to dissect their temporal dynamics. To date, neuropsychiatric-like phenotypes have not been characterized in depth in the genetic rat models described here, and the results obtained so far require further corroboration. Research on these PD genetic rat models hardly focused on apathy and impulsivity-related behaviors, although altered

motivation has been indirectly suggested in α-synuclein BAC rats, based on a faster decline in activity and a decreased exploration of the central zone of an automated cage apparatus over time, along with suppressed feeding[170]. Regarding depression, *DJ-1* KO rats show signs of behavioral despair by 6 months[167], and in *PINK1* KO female rats, there is evidence for anhedonia by the age of 8 months, whilst *PINK1* KO males were not assessed simultaneously[169]. In the α-synuclein BAC rats, both increased and decreased anxiety-like behaviors have been reported[84,201]. In the same rats, locomotor activity is enhanced in a novel environment by 3 months of age, and deficits in prepulse inhibition emerge as well at a more advanced age[201]. Both behavioral features have been associated with psychosis-like behavior in rodent models[202]. It is worth noting that the psychosis-like phenotype is stronger in α-synuclein BAC male rats relative to females, in agreement with evidence for sex differences in the PD symptomatology in patients[203]. This supports the assessment of sex differences in psychosis in the human population.

A significant percentage of PD patients suffer from a mild cognitive impairment which can convert into dementia with disease progression[196,199]. Cognitive deficits in early PD stages commonly impact several facets of executive functioning, visuospatial skills and memory and have been related to dysfunction in multiple neurotransmitter systems as well as common PD neuropathological alterations[199]. Analyses of some cognitive components have been performed in lesion rat models of PD, which present though some limitations in terms of cognitive phenotypes that can be reproduced[204,205]. On the contrary, cognition has rarely been investigated in PD genetic rat models. *PINK1* KO rats display normal recognition and spatial memory when tested at 3 months[206]. *DJ-1* KO rats were found to have altered short-term memory by 4.5 months, but unchanged goal-directed behavior[166,167]. Changes in short-term memory were also observed in *DJ-1* KO mice, but at a later age compared to *PINK1* KO rats[207]. Although it may not reflect the deficits in patients, the early rat phenotype is more consistent with the early appearance of cognitive deficits in human symptomatology, if the same temporal dynamics also apply to the familiar PD forms. In the α-synuclein BAC rats, knowledge of cognitive aspects is very limited.

In summary, all three PD rat models reflect, to a certain extent, the motor impairment in the disease. *DJ-1* KO and *PINK1* KO rats are ideal for reproducing cranial sensorimotor deficits and studying the underlying mechanisms. The α-synuclein BAC rats mimic the olfactory dysfunction and specific psychiatric features of the disease, but cognition remains scarcely examined in any of these models. Apathy, a frequent symptom in PD patients, has not been sufficiently investigated in genetic rat models of PD. Moreover, tremor, a main motor feature in the disease, does not appear to be reproduced in genetic rat models.

Behavioral phenotypes in genetic rat models of Huntington's disease: tgHD and BACHD rats

HD patients present motor impairment, cognitive deficits and psychiatric manifestations[208]. The tgHD and BACHD genetic rat models mimic many of these HD behavioral features. Compared to mouse fragment models, especially R6/2 mice, the phenotype in tgHD rats develops later and progresses at a slower pace[108,209]. Motor impairment starts earlier and has faster progression in BACHD rats compared to tgHD rats, with the first BACHD rat motor abnormalities starting at the age of 1 month[107] and the motor deficits in tgHD rats beginning at about 6 months[210]. In the tgHD rat model, phenotypes appear stronger in homozygous compared to hemizygous animals[210] and male rats were reported to be more sensitive to motor coordination impairment relative to females[211], while in the BACHD rat model, homozygous females seem to develop a stronger motor, emotional, and cognitive phenotype than males[212], although information on sex differences and homozygous animals in this model is still limited.

In general, the tgHD and BACHD rat models exhibit reduced motor coordination and balance[107,108,210,211,213,214], altered locomotor activity and rearing[107,211,213,215,216], decreased muscle endurance[215,217]

and gait abnormalities[107,213,218]. At late time points, tgHD rats are also affected by choreiform neck movements which are more frequent in homozygous individuals[219]. Prepulse inhibition of the startle response, a measure of sensorimotor gating, is decreased in HD patients[220]. In BACHD rats, there are mild sensorimotor gating deficits at the age of 9 months[213], whereas in tgHD rats, no sensorimotor deficits have been detected[216,221].

Emotional and behavioral symptoms in HD patients can precede motor symptoms by decades. A variety of psychiatric symptoms characterize the disease where apathy, depression, irritability, aggression, and anxiety are frequently reported[222]. Likewise, cognitive deficits in HD patients can be found several years before motor diagnosis[223] and are heterogeneous, embracing problems with executive function, visuomotor integration, psychomotor speed, and social cognition[224-227]. While the available tests in rodents can only partially assess the multidimensional nature of the neuropsychiatric disturbances in HD patients, emotional changes have been shown with different behavioral paradigms in HD rat genetic models. Both tgHD and BACHD rats show a low anxiety phenotype in different behavioral setups[107,108,210,211,214,228]. In tgHD rats, the emotional phenotype is already detectable at the age of 1 month, before motor deficits[210], whilst motor and emotional alterations in BACHD rats follow the opposite temporal pattern[107]. In BACHD rats, evidence for increased anxiety-like behavior was also found in specific paradigms[229], in line with human data. The contradictory anxiety phenotype remains mostly unexplained, although it could in part be dependent on age and on the different components of anxiety targeted by different typologies of behavioral tests which could in turn rely on distinct brain mechanisms. One study demonstrated that the disinhibition of the central nucleus of amygdala via $GABA_A$ receptor antagonist in BACHD rats increased avoidance and escape responses in an avoidance task as well as the social exploration in a social test[230], implicating an altered activity in the central nucleus of the amygdala as one of the mechanisms at the base of anxiety-related behavioral alterations. Further investigations of emotional phenotypes in tgHD rats revealed enhanced emotional learning in discriminative Pavlovian fear conditioning and hyperreactivity to aversive emotional events which were paralleled but not explained by shrinkage of the central nucleus of the amygdala[217].

Depression-like behavior reported in multiple studies in HD fragment and full-length mouse models[231-234] has not been studied in much detail in HD rat genetic models. An impaired hedonic reaction in response to sucrose in tgHD rats has been associated with anhedonia-like behavior[217] which was though not confirmed by later analyses[228]. BACHD rats show decreased sucrose preference at 3 months and this effect is maintained at later time points[235]. Along with hedonic deficits, BACHD rats present impaired reward-directed behavior by the age of 3 months[235], indicating a lack of motivation which could be representative of apathy, a core symptom of HD[223]. However, the BACHD rat shows notable obesity, and it is currently uncertain how that might interact with behavioral tests that are based on food rewards. Still, there do seem to be some indicators of the animals putting a lower hedonic value on small reward pellets[236,237].

A key cognitive impairment in HD is executive dysfunction. One of the main executive function deficits is impaired inhibitory control, which can be detected in specific behavioral tests in HD patients[238,239]. It was also shown in HD fragment and knock-in mouse models[240,241] and in transgenic rats[242-245]. Rat models, in general, may be advantageous over mouse models in the applied paradigms and have been largely used in preclinical research on impulsive control[246]. Impulsive-like behavior in tgHD rats was detected in both sexes at 15 months and with different strain backgrounds[243,245]. Deficits consistent with the inability to withhold inappropriate lever responses have been shown in BACHD rats already by the age of 3-4 months[242,244]. tgHD and BACHD rats further mimic several other facets of cognitive dysfunction in HD patients[223,247,248]. Deficits in both animal models were reported at different ages depending on the cognitive aspect considered. In both BACHD and tgHD rats, the first cognitive deficits were found early, at 3 and 4

months, respectively. In tgHD rats, cognitive deficits concern, among others, cognitive flexibility, attention, working memory, visuospatial and visual object memory, temporal perception and psychomotor performance[210,219,221,249-251]. BACHD rats show impaired reversal learning[111,214,252], deficits consistent with fronto-striatal dysfunction in different short-term memory tests[253], decreased performance in a decision-making task[254] and impaired associative memory[252].

Several aspects of social behavior and social cognition are abnormal in HD patients who face problems with emotion recognition and awareness as well as theory of mind and, to a certain extent, empathy, which have been associated with altered social skills and self-reported social distress[224,255-257]. Transgenic fragment and full-length HD mouse models display changes in various social behavior parameters[258-262]. Compared to mice, rats show lower group aggression[4] and are more interested in the interaction with male conspecifics[18]. Therefore, free social interaction experiments in males can be better performed in rats. Both male and female tgHD rats tend to interact more than wild-type rats with the same sex conspecific starting from 1 or 2 months of age, which was interpreted as a low anxiety-like phenotype[210,211]. An automated analysis of the BACHD rat behavior in a social interaction test between 2 and 8 months of age demonstrated alterations in multiple social interaction parameters[263]. Other analyses in the model further revealed changes in other areas of social cognition[229,263]. It is difficult to draw direct parallels between social behavior parameters measured in humans and rats as social behavior is highly species-specific. Nevertheless, given that brain correlates of social behavior are under several aspects comparable in humans and rodents[264], it is still reasonable to model main social behavior related functions in rats. Depending on age, in the BACHD rats, we find a more aggressive play behavior, decreased tendency to search for or interact with a conspecific and a decreased social preference[229,263], which in part indicates higher anxiety and may altogether be representative of a disrupted socio-cognitive function. It would then be important to relate social behavior alterations to changes in brain areas relevant to social behavior. In the BACHD rat model, in addition to the evidence for an involvement of the amygdala in the modulation of anxiety in a social context[230], a decreased BDNF gene expression was also reported in the ventral striatum[263]. While the striatum does not have a primary social function, it has been suggested to integrate social information into main striatal functions, like reward[265]. Future analyses could consider assessing the expression of markers relevant to social behavior, such as oxytocin and vasopressin[265,266], and focus on other brain areas affected in HD, like the hypothalamus, which shows changes in neuronal populations expressing these markers[267]. In HD patients, cerebrospinal fluid oxytocin levels were also found to be decreased and to correlate with social cognitive scores[268]. As part of social behavior, aggression is often reported in HD patients[255], but has not been assessed in transgenic rat models. While analyses of aggression could take advantage of well-established tests in rats, they may be sensitive to the model strain, which adds to the complexity of a phenotypic profile.

Altogether the BACHD and tgHD rat models reproduce many features of the HD triad of symptoms. Both models present motor and cognitive deficits, and some have been reproduced across studies. These rat models also display emotional alterations. The bidirectional anxiety phenotype in the BACHD rat model supports further assessments, especially in terms of underlying mechanisms. Furthermore, several phenotypes in the HD rat models and in the models of other neurodegenerative disorders have been assessed only once. Thus, their repeatability must still be determined. In addition, it remains largely unclear to what extent specific phenotypes in animal models and similar symptoms in humans share the same biological mechanisms, thereby representing the same kind of impairment.

PHENOTYPIC/ASPECT-REPLICATING MODELS TO STUDY AD, PD, AND HD

There is still a vast gap between preclinical studies to effective treatments for patients[269-271]. To date, translatability from animal models to humans in terms of treatment efficacy, adverse effects, and tolerability

has been found to often not correlate[272-274]. And likewise, a proportion of unknown size of therapeutics fails to enter the clinic, being not beneficial in animal models, though they might be effective in humans.

Despite the discouraging success rates in finding new therapies for NDs, rats have been essential for discerning many aspects of neurological functions. However, with the more readily genetic manipulation of mice and the discovery of many disease-causing genes for NDs, mice have outnumbered rats in studies evaluating behavioral aspects of neuroscientific questions in the last two decades[275]. Also, in studies describing therapeutic approaches in AD, PD, and HD, this trend towards using mice is reflected by the number of publications listed in PubMed [Figure 1].

Preclinical studies require a model to present a phenotype that is robust, fast developing, replicating key aspects of the human disease, and compatible with the form of treatment investigated. Some aspects of human disease are, however, only ever hardly modeled in animals. As one important example, cell loss is often not found in genetic models of neurodegeneration or only towards the end of the lifespan. Additionally, genetic rat models often display milder phenotypes than mouse models when based on the same construct, and these phenotypes often take relatively long to develop[276]. Therefore, we briefly describe in this section models with induced cell loss - though fairly artificial - which have helped to model neuronal demise and to evaluate therapies that can halt or even reverse this process. Commonly used models, with such induced neurodegenerative phenotypes, are summarized in Table 2. Their fast-appearing nature and cost-effectiveness, in comparison to generating new genetic rat lines, make them a resource to be relied upon frequently.

Phenotypic rat models of Alzheimer's disease

AD poses a challenge for finding appropriate models, because sporadic cases caused by mutations in AD-risk genes outnumber familial cases[285]. While rats are genetically closer to humans in terms of tau isoforms, rats seem to be more resistant to developing characteristic neuropathological features of AD when expressing human genes. They present fewer plaques and tau tangles are not present[67,276]. Injection of neurotoxins or overexpressing constructs of Aβ into the brain are commonly used to induce local cell death and to model the AD typical neuropathology. For this, the larger brain size of the rat offers advantages over mice, as stereotactic injections can be performed more consistently and with larger volumes. Additionally, these models have been mostly used in preclinical studies.

Rats with diminished cholinergic neuron populations or severed neuronal circuits show memory deficits and impaired learning[278], thereby resembling the cognitive symptoms observed in patients, but not the pathobiological origin of the disorder. Ibotenic and okadaic acid, amongst other cholinergic neuron harming compounds, or surgically lesioned rats, have been used to study neuroprotective or even regenerating therapies. Exemplary, with these phenotypic models, it was possible to demonstrate that neuronal stem cells or mesenchymal stem cells can replace or protect cholinergic neurons and improve spatial learning and memory[286-288]. Recapitulating early pathobiological events, Aβ-injected models have also been used to investigate the beneficial effects of stem cell transplantation[289-291]. It should be noted in this regard that the concentrations needed to induce the pathological phenotype by Aβ-injections exceed any physiological concentrations, and the stereotactic injections always produce unwanted tissue damage at the injection site. Genetic mouse models of AD have been used to elucidate the mechanisms underlying the observed amelioration in the genetic context of AD[292,293]. A meta-analysis of preclinical studies on stem cell therapy for AD found a large variation in the models used and origin of cells but concluded overall beneficial effects on memory and learning. Approximately 60% of the analyzed studies were performed on non-genetic rat models[294].

Table 2. Commonly used phenotypic models of NDs

	AD		AD/PD	PD	HD	
	Physical/chemical lesion of cholinergic centers	**Aβ injection**	**LPS**	**6-OHDA**	**QA**	**3-NP**
Aspect of disease reproduced	Degeneration of cholinergic neurons	Memory deficits behavioral alteration Neuroinflammation Aβ accumulation Local cell loss	Neuroinflammation cognitive deficits Aβ and tau accumulation Sickness behavior	Dopaminergic cell loss, lesions Sensitivity to apomorphine Neuroinflammation	Striatal lesions Behavioral alterations Excitotoxicity-induced cell loss	Striatal neurodegeneration of MSN Motor deterioration and behavior alterations impairs mitochondrial energy production
Acute or progressive?	Acute	Single injection (acute) Osmotic pump (progressive)	Acute, severity can be modulated by the amount of LPS challenges	Acute, compensatory mechanisms possible	Acute, chronic	Progressive over multiple injections
Pros	• Different protocols available • Easy to implement • Systemic injections are possible with some chemicals	• Rapid appearance of Aβ accumulation	• Aspects of neuroinflammation can be studied • Systemic injections	• Lesion intensity can be modulated • Dopaminergic neurons are targeted	• Similar to the pattern of cell loss in HD patients	• Systemic injections • Histological similarities to HD
Cons	• Many variables (age at lesioning, size/type of lesion, strain, *etc.*) • Limited to the lesioned brain area • No Aβ or tau pathology	• High concentration needed • Aging as a pathological factor neglected • Brain injury	• No AD/ PD-specific pathology	• Variability within animals • Compensatory effects in unilateral lesions	• Many variables (age at lesioning, size/type of lesion, strain, *etc.*)	• High inter-animal variability in lesioning • Many variables (age at lesioning, size/type of lesion, strain, *etc.*)
Literature	Reviewed in[277]	Reviewed in[277,278]	Reviewed in[279]	Reviewed in[280]	Reviewed in[281-283]	Reviewed in[284]

AD: Alzheimer's disease; PD: Parkinson's disease; HD: Huntington's disease; Aβ: amyloid beta (Aβ) peptide; LPS: lipopolysaccharide; 6-OHDA: hydroxydopamine; 3-NP: 3-nitropropionic acid; QA: quinolinic acid.

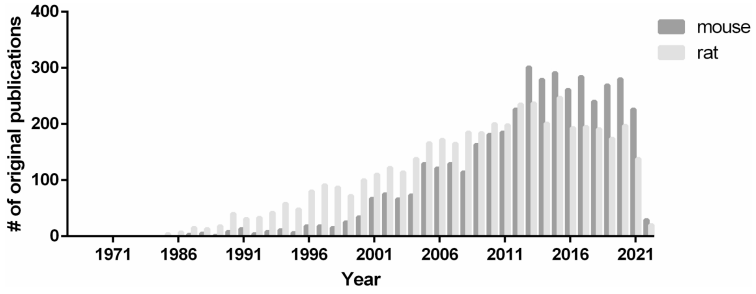

Figure 1. Studies referencing mice or rats in therapy approaches for Alzheimer's, Parkinson's and Huntington's disease. Results of PubMed search with terms "mice"/"mouse" and "rat"/"rats" in combination with above mentioned neurodegenerative disorders and "therapy". Review articles have been removed from the year in which they were published.

Aside from lesion models, what needs to be noted in AD and PD are lipopolysaccharide (LPS)-induced models, as chronic inflammation is associated with cognitive impairment in AD patients and the exacerbation of AD and PD pathology in models of the disease (reviewed in[295,296]). These models recapitulate the involvement of the immune system in the pathogenesis and show an increase in Aβ and phosphorylated tau and cognitive impairment[279,297,298].

Over the past decade, improvements in biomarker identification and quantification and improved preclinical study design have been implemented to increase translatability to human studies. Incorporating such study design, a genetic rat model has been used in a preclinical study with improved longitudinal assessment of biomarkers to improve translatability. Continuous CSF and plasma collection for measurement of Aβ and neurofilament light chain in combination with PET and MRI imaging have been used to evaluate an anti-amyloid therapy in McGill-R-Thy1-APP transgenic rats[299].

Phenotypic rat models of Parkinson's disease

Due to the multifactorial etiology of PD and most cases being of idiopathic origin, neurotoxin and lesion models are mostly relied on for preclinical Parkinson's research. The main neuropathological feature of the disease, the loss of dopaminergic neurons in the substantia nigra can be modeled through the injection of hydroxydopamine (6-OHDA) in most studies into the substantia nigra pars compacta or in the medial forebrain bundle[300]. Next to cell loss, lesioned rats show motoric deficits that are correlated to the degree of dopaminergic neuron loss, oxidative stress, and neuroinflammation[280,301]. Test paradigms have been developed to assess motor deficits, resembling akinesia, fine motor impairment, and showing rotational response to dopaminomimetic agents when extensive unilateral lesioning is produced[301]. While the lesions produced resemble cell loss in humans, unilateral lesions are mostly used in experimental settings, inducing cell loss in one hemisphere only. These lesions are mostly produced in rats, as mice are more prone to weight loss and post-lesion mortality which can be circumvented by modification of the injection sites and improved post-surgical surveillance[302-304].

Another neurotoxin model is the MPTP mouse model. In contrast to 6-OHDA, which does not cross the blood-brain barrier, MPTP can be administered systemically, but shows larger variation in neuronal loss in the substantia nigra and the motor phenotype is not fully equivalent to PD patients[305]. MPTP has been mainly used to mimic PD in mice in many different treatment studies, as rats are highly resistant to MPTP. One rat model of unilateral brain infusion with MPP+ has been developed, which shows progressive loss of dopaminergic neurons[306].

Phenotypic rat models of Huntington's disease

Only few preclinical studies have been performed in transgenic rat models of HD despite the monogenetic etiology of HD[111,307]. To a greater extent, neurotoxin models are used to model histopathological characteristics of the disease or mechanism of neuronal demise to test preventive therapies or therapies aiming at restoring functionality. The two most commonly used substances are quinolinic acid (QA) and 3-nitropropionic acid (3-NP). QA is an excitotoxin, binding to the N-methyl-d-aspartate (NMDA) receptor and more strongly affecting neurons within the hippocampus, striatum, and neocortex. It can induce different neuron and glia-damaging effects, also dependent on the dosage[308]. The lesions produced are structurally similar to HD characteristic lesions within the striatum and limited to the area around the injection site[283,309]. Impairment of paw use can be assessed in cylinder test, altered grooming behavior has been described, and learning and motoric abilities are altered in this model[310-312].

Systemic injection with 3-NP, an irreversible inhibitor of succinate dehydrogenase in the mitochondria, leads to striatal neuronal degeneration, as well. Rats are more sensitive to 3-NP than mice and develop lesions and behavioral alterations[284,313]. The lesions produced by 3-NP are more severe and cause a phenotype that includes learning impairment, reduced grip strength, and balance deficits that are more severe than in the QA model[310].

In current treatment approaches and clinical trials, HTT is lowered independently of the mutation or in an allele-selective manner[314]. Preclinical studies lowering HTT by micro-RNA (miRNA) have been performed in genetic mouse models of HD, an acute rat model of HD, a large animal model, and non-human primates[315-317]. Acute and local expression of HTT by lentiviral- or adenoviral vectors produces models that replicate typical neuropathological features HD, like aggregation and neuronal dysfunction[315,318,319]. This rat model can be used to evaluate the HTT lowering effects before a long preclinical trial is initiated, for example, by investigating behavioral readouts. Most allele selective therapies utilize heterozygous single nucleotide polymorphisms (SNPs) that are associated with the mutation-carrying allele. These therapeutic targets are only found in fractions of a population, and accordingly, they are also not necessarily present in the constructs that have been used to generate genetic models. Therefore, acute rat models can be used to test combinations and variations of SNP targeting molecules to advance personalized therapies.

CONCLUSION

Huge strides have been made towards generating genetic rat models in the past 20 years. These genetic models are an important asset for research on NDs to study physiological and pathophysiological mechanisms. Rats add to the functional understanding of disease by allowing electrophysiological measurements, harvesting of primary cell cultures and a wider range of surgical procedures. They offer the possibility to evaluate therapeutic effects more precisely due to their genetic similarities to humans, larger body size compared to mice, and the associated possibility of multiple sampling of biofluids over time. Many behavioral tests have been developed in rats, enabling a more robust assessment of behavioral phenotypes in rat models. Moreover, rats display a more complex behavioral repertoire than mice, allowing more sophisticated extrapolation to the human condition. Often efforts are being made to provide a complete characterization of the models, offering a good starting point to find an adequate fit for the biological question to be answered. Despite the long list of advantages rats offer, they are less represented in biomedical studies than mice. One reason for this is that genetic models have been generated with a delay due to the technically challenging manipulation of the rat genome. This, economic reasons, and the multifactorial etiology of many NDs have made phenotypic rat models commonly used models in preclinical research. Still today, they fill a gap when genetic models cannot reproduce certain aspects of disease, highlighting that in most cases only a combination of readouts, models, and model species can answer biomedical questions adequately. New rat models have been developed and characterized recently and can offer additional insight into disease mechanisms. Whether rats as models, combined with improved study design, can increase the translational value of biomedical research remains to be seen.

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Authors' contributions

Drafting, writing, revision of manuscript: Novati A, Singer-Mikosch E, Yu-Taeger L, Clemensson E, Nguyen HP

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Ageing and Neurodegenerative Diseases

Review

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Oxidative stress-mediated inflammation promotes the pathogenesis of amyotrophic lateral sclerosis

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Abstract

Neuroinflammation in amyotrophic lateral sclerosis (ALS) is characterized by activation of monocytes/macrophages and T lymphocytes in the periphery and microglia and astrocytes within the central nervous system. This review emphasizes the role of oxidative stress in promoting systemic inflammation and the early stages of neurodegeneration. Motor axon terminals of ALS patients have significantly increased intraluminal calcium and dysfunctional mitochondria, increasing the formation of lipid peroxides and ferroptosis programmed cell death. Serum lipid peroxides and acute phase proteins are elevated, and regulatory T lymphocytes (Tregs) are dysfunctional, impairing immune-mediated neuroprotection. Macrophages are pro-inflammatory; the expression of genes involved in inflammation is increased in peripheral monocytes/macrophages of ALS patients. Suppressing these multiple components of inflammation is an important therapeutic goal and provides an opportunity to interrupt the self-propagating cytotoxic cycle. Two clinical trials with autologous infusions of ex vivo expanded Tregs have been safe and well tolerated, with promising clinical results associated with suppression of pro-inflammatory lipid peroxides.

Keywords: Amyotrophic lateral sclerosis, ferroptosis, oxidative stress, lipid peroxides, 4-hydroxynonenal, oxidized LDL, acute phase proteins, regulatory T lymphocytes

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by relentless degeneration of upper and lower motor neurons. Following symptom onset, patients survive an average of 3 to 5 years. The widespread use of non-invasive ventilation, early attention to proper nutrition, and promotion of safe exercises and prevention of falls have made a difference in patients' quality and length of life, but the ability of therapy to significantly modify the pathogenesis of the disease is still limited. Although the cause(s) and pathogeneses of ALS are still to be completely defined, advances in gene sequencing have led to the discovery of mutant genes that cause ALS, many of which encode proteins that compromise immune function. In fact, linkage of these mutations to ALS provides the most cogent evidence that immune dysregulation contributes to the pathogenesis of ALS[1]. Even in 90% of ALS patients without a positive family history of the disease, innate and adaptive immune cells are pro-inflammatory and modulate motor neuron injury and disease progression.

In ALS, motor neuron cell injury and death are initiated by multiple cell-autonomous pathways leading to misfolded proteins, mitochondrial dysfunction with increased intramitochondrial calcium, oxidative stress, impaired autophagy and altered RNA metabolism[1]. In ALS transgenic mouse models, injured motor neurons interact with surrounding glia and peripheral and central immunomodulatory cells, which receive the message to protect and repair. The initial glial and immune reactivities are neuroprotective[2]. However, as the intraneuronal injury process continues, the message from the motor neuron changes. The new message promotes a pro-inflammatory cascade. Most investigations of neuroinflammation have focused on the central nervous system (CNS), where microglia and astrocytes are pro-inflammatory; neuroprotection is impaired, and release of pro-inflammatory cytokines promotes further injury to the motor neuron. However, it is becoming increasingly clear that peripheral inflammation may contribute significantly to motor neuron injury and cell death[3]. In the following sections, the factors that initiate and sustain both peripheral and CNS inflammation are reviewed, and our clinical efforts to slow the evolving pathogenesis of disease in ALS patients are described.

PATHOLOGICAL CHANGES IN MOTOR AXON TERMINALS

The motor neuron projects outside the blood-brain barrier to the muscle and the neuromuscular junction may be one of the early sites of pathology, initiating a "dying back" from the neuromuscular junction[4]. To determine the potential contribution of motor neurons in initiating widespread oxidative stress, we used electron microscopy to examine the ultrastructure of ALS patient motor nerve terminals in muscle biopsy specimens. Seven ALS patients, ten non-denervating disease control subjects, and five patients with denervating neuropathies were studied[5]. Following oxalate-pyroantimonate fixation to preserve in situ calcium distribution, we noted swollen calcium-containing mitochondria, increased density of synaptic vesicles, increased active-zone vesicle density, and increased intraluminal calcium precipitates within membranous organelles. These changes were not present in either denervating or non-neuropathic controls. There was minimal Schwann cell envelopment of the ALS motor terminals compared to the neuropathic controls, possibly due to impaired neuronal Schwann cell signaling [Figure 1].

At the time we published these findings, the concept of ferroptosis as a significant pathway of programmed cell death had not been recognized. In 2012, ferroptosis was described as a cause of cell death distinct from apoptosis, necrosis, and necroptosis[6]. Ferroptosis is now recognized as a form of iron-dependent regulated cell death driven by lipid peroxidation. Polyunsaturated fatty acids (PUFAs), such as arachidonic acid within phospholipid-containing membranes, undergo peroxidation, which yields neurotoxic moieties such as 4-hydroxynonenal (HNE). Free PUFAs are not themselves drivers of ferroptosis and are not intrinsically toxic. It is the accumulation of oxidized PUFA-containing lipids within cell membranes that drives lipid

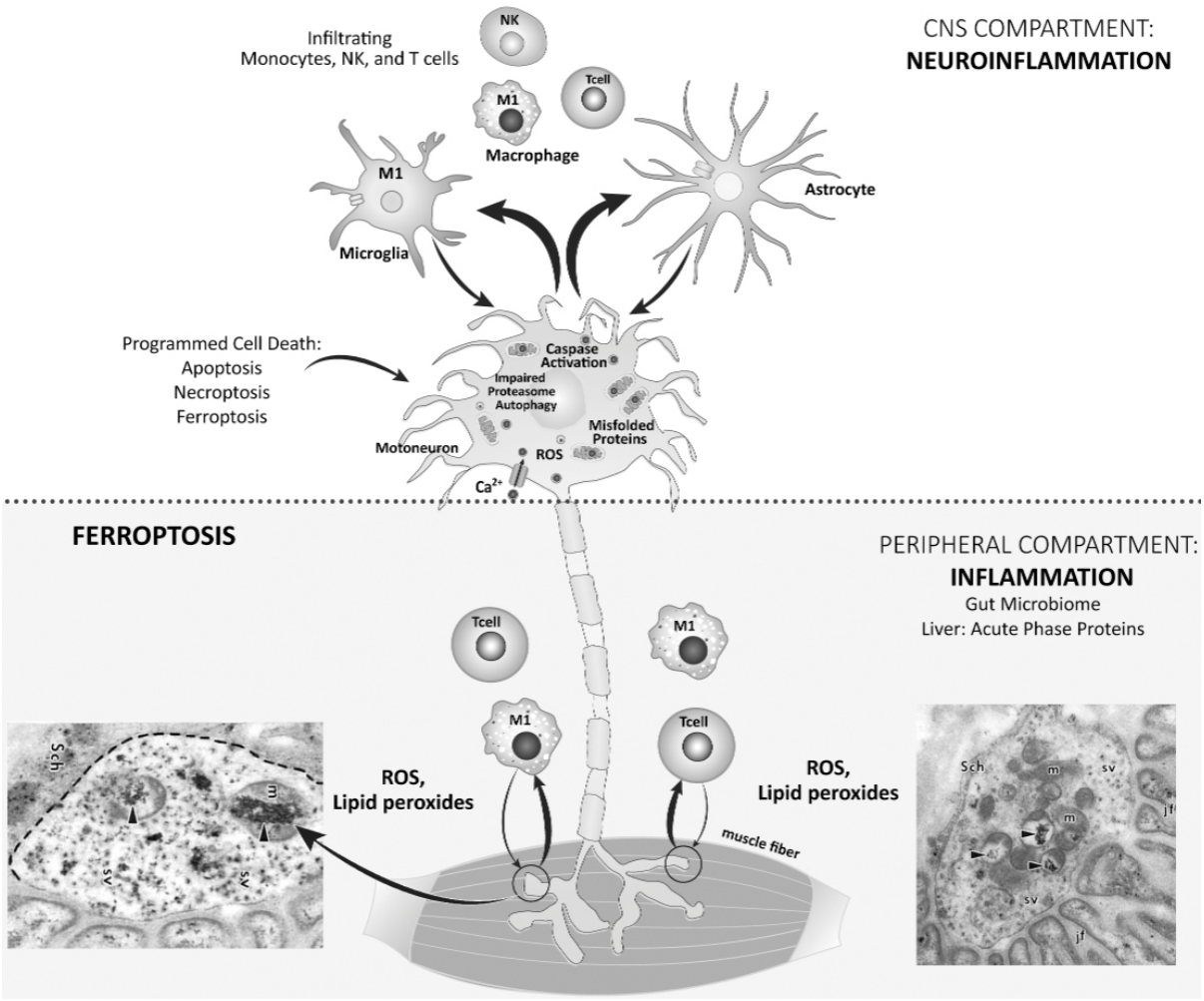

Figure 1. Systemic inflammation is initiated in motor axon terminals of the peripheral compartment In ALS patients. On the lower panel L- and R-sides we present ultrastructural illustrations of terminals from two different ALS patients[5]. Calcium precipitates are present in intraluminal membrane bound organelles and mitochondria. The dysfunctional mitochondria induce reactive oxygen species (ROS) and lipid peroxides, and promote ferroptosis, which activates macrophages and T lymphocytes. In turn, the activation by oxidized lipids induces macrophage production and secretion of IL-6, IL-1β, and TNF-α, followed by synthesis and release of acute phase proteins by the liver. The gut microbiome directly interacts with the immune system at this early stage. In the upper panel neuroinflammation in the CNS compartment is the consequence of "dying back" from the axon terminal as well as the spread of pro-inflammatory immune cells from the periphery to the CNS. The spread of pro-inflammatory immune cells from the CNS back to the periphery then promotes a self-propagating cytotoxic cascade. The result is activation of programmed cell death pathways involving apoptosis, necroptosis, as well as ferroptosis (Modified from Figure 1, Ref. 3[3]). ALS: Amyotrophic lateral sclerosis; CNS: central nervous system.

peroxide formation and ferroptosis.

The most basic characteristics of ferroptosis are iron overload and a lethal accumulation of intracellular lipid peroxides and reactive oxygen species (ROS)[7]. The peroxidation of membrane-bound PUFA-containing lipids is driven by both the labile iron pool facilitating the Fenton reaction, which propagates non-enzymatic lipid peroxidation[8], and by iron-dependent enzymes, such as arachidonate lipoxygenases, that initiate the formation of lipid hydroperoxides as substrates for the Fenton reaction[9]. The significantly elevated serum levels of HNE, as well as the increased levels of ferritin and decreased levels of transferrin as reported in a meta-analysis review, provide evidence for ferroptosis as a cell programmed cell death pathway in ALS[10]. Other sources of iron result from the compromised blood-brain barrier and the entry of

hemoglobin into the CNS parenchyma[11]. Microvascular lesions reported in SOD1G93A mice contain blood-derived hemoglobin that releases free iron, which can catalyze the formation of neurotoxic free radical species[12]. Ferroptosis is also immunogenic and can promote inflammation[13]. Ferroptosis promotes the release of inflammatory factors and damage-associated molecular patterns, enhancing the pro-inflammatory environment, inducing inflammation by releasing IL-33, and activating additional pathways[14]. Ferroptosis has also been reported to accelerate the metabolism of arachidonic acid, which stimulates the synthesis of bioactive inflammatory mediators such as prostaglandins and leukotrienes.

Mitochondrial changes in ALS are not limited to motor axon terminals. In ALS mouse models and ALS patients, motor neuron mitochondria are dysfunctional and intracellular calcium is dysregulated[15]. Mitochondria are reported as fragmented. Calcium homeostasis is impaired, and respiratory chain activity and ATP synthesis are decreased. Levels of glutathione (GSH) are significantly reduced in ALS. In one study, GSH levels were reduced to 22.25 ± 0.99 μg/mL, compared to controls 131.54 ± 12.05 μg/mL[16]. With significantly reduced levels of GSH, mitochondria can initiate or enhance cell susceptibility to ferroptosis by promoting lipid peroxidation or enhancing ROS production. Ultimately, lipid peroxides spread to the plasma membrane, where they trigger the rupture of the plasma membrane and cell death. The electron transport chain may also be involved secondary to the leakage of electrons that produce superoxide and H_2O_2, which can then react with Fe (II) to drive Fenton chemistry and non-enzymatically-mediated lipid peroxidation. Ultimately the significantly increased levels of lipid peroxides provide the most compelling evidence for ferroptosis-mediated programmed cell death in ALS.

MONOCYTES/MACROPHAGES

Innate immune myeloid cells are the first line of defense, mediating both anti-inflammatory and pro-inflammatory functions. Monocyte/macrophage myeloid cells are often designated as anti-inflammatory M2 or pro-inflammatory M1. However, myeloid cells do not exist either as M2 or M1 but are an overlapping continuum from anti-inflammatory to pro-inflammatory phenotypes. A similar state exists within the CNS where innate immune cells, namely microglia, can express a continuum of anti- or pro-inflammatory states. Monocytes can be differentiated from inducible progenitor stem cells and can then be differentiated into anti-inflammatory or pro-inflammatory macrophages[17]. The anti-inflammatory macrophages are neuroprotective in vitro and suppress pro-inflammatory cytokine signaling, while the pro-inflammatory macrophages are neurotoxic in vitro and enhance pro-inflammatory cytokine synthesis and secretion. Thus monocytes have the potential to be protective or toxic, with the phenotype dictated by environmental signaling.

In ALS mouse models, microglia are initially neuroprotective in the early stages of the disease and subsequently transit to a pro-inflammatory state[18]. The specific molecular determinants of this transition are far from clearly understood but appear to derive from communications between the motor neuron projections outside the blood-brain barrier at the neuromuscular junction and the peripheral immune cells. Within the CNS, the dialogue is between injured motor neurons and glia. There is also a continual dialogue between peripheral and CNS compartments. Neuroprotection and neurotoxicity are overlapping responses of myeloid populations to signals initiated by motor neurons which may vary with intensity of injury. Both peripheral and central compartments become readily involved as immunomodulatory signaling spreads from the periphery to CNS and from CNS back to the periphery.

In ALS patients, an understanding of the specific myeloid phenotypes at the earliest stages of the disease is presently limited. We can only determine the phenotypes after the disease process is underway, and then primarily in the peripheral compartment. In ALS, serum monocytes are activated and pro-inflammatory. To

define the pro- or anti-inflammatory signaling of peripheral circulating monocytes in an unbiased manner, we used high-throughput deep RNA sequencing (RNA-seq)[19]. Our deep RNA-seq and qRT-PCR data demonstrated that monocytes isolated from patients with ALS expressed a unique genetic profile associated with pro-inflammatory immune responses [Figure 2]. Interleukin-1β (IL-1β), interleukin-8 (IL-8), nicotinamide phosphoribosyltransferase (NAMPT), FosB proto-oncogene-AP-1 transcription factor (FOSB), and CD83 were prominent upregulated disease-related genes involved in ALS monocyte-mediated pro-inflammatory responses. IL-1β is a major inflammatory cytokine produced by inflammasome activation in monocytes/macrophages. Blockade of the IL-1β receptor decreases microglial activation, reduces motor neuron loss and prolongs survival in ALS mice[20]. NAMPT promotes interleukin-6 (IL-6) production. Silencing *NAMPT* gene expression in monocytes reduces IL-6 production, decreases T helper 17 cells and decreases infiltration of monocytes/macrophages[21]. CD83 is involved in regulating antigen presentation, and in monocytes of rapidly progressing ALS patients, CD83 promotes upregulation of antigen presentation. The expressions of several cytokines and chemokines, namely, IL-8, CXC motif chemokine ligand 1 (CXCL1), and CXCL2, are increased in $CD14^+$/ $CD16^-$ ALS monocytes and promote migration to sites of inflammation[22]. Peripheral monocytes/macrophages and lymphocytes infiltrate the CNS and can combine with central inflammatory responses to promote a self-propagating amplification of inflammation and injury.

Our unbiased investigation clearly documents the pro-inflammatory phenotype of ALS monocytes. Although several studies assert that pro-inflammatory cytokines are elevated in the serum of ALS patients, the reported results from ELISA assays are quite variable. However, a meta-analysis did report that the pro-inflammatory cytokines IL-6, IL-1β, and tumor necrosis factor-α (TNF-α) are increased in ALS blood[23]. The increased gene expression of inflammatory markers adds to the compelling evidence that ALS monocytes are skewed toward a pro-inflammatory phenotype and could influence rates of disease progression; monocytes of rapidly progressing patients expressed more inflammation-related differentially expressed genes than slowly progressing patients.

Our further studies documented that ALS monocytes are more activatable than monocytes from healthy controls[24]. We differentiated peripheral monocytes into pro-inflammatory and anti-inflammatory macrophages and determined their gene expressions. ALS pro-inflammatory-derived macrophages produced more pro-inflammatory cytokines than healthy control pro-inflammatory-derived monocytes. IL-6 mRNA and TNF-α mRNA expressions were significantly increased in ALS macrophages, as were IL-6 and TNF-α proteins, compared to healthy control activated macrophages. The increased IL-6 protein correlated with the burden of ALS disease, and TNF-α protein correlated with rates of disease progression. Collectively these data document the loss of macrophage-mediated neuroprotection and the predominant neurotoxic pro-inflammatory phenotype of ALS monocytes/macrophages.

The factors initiating the pro-inflammatory phenotype of peripheral monocytes/macrophages in ALS have not been clearly delineated. The presence of significantly increased levels of HNE lipid peroxides as well as oxidative stress could drive the activation of peripheral monocytes/macrophages, enhancing the synthesis and release of pro-inflammatory cytokines[5]. In turn, the activation of macrophages can enhance the production of free radicals and the synthesis of HNE [Figure 3]. The ferroptosis-mediated lipid peroxide synthesis in ALS motor axon terminals could be an initiating event, but this remains to be proven. The gut microbiome could also be an early event initiating the self-propagating cycle of HNE-pro-inflammatory cytokine reactivity[3]. Suppressing ferroptosis or the resulting pro-inflammatory myeloid cells certainly should represent an important component of any disease-modifying therapy for ALS.

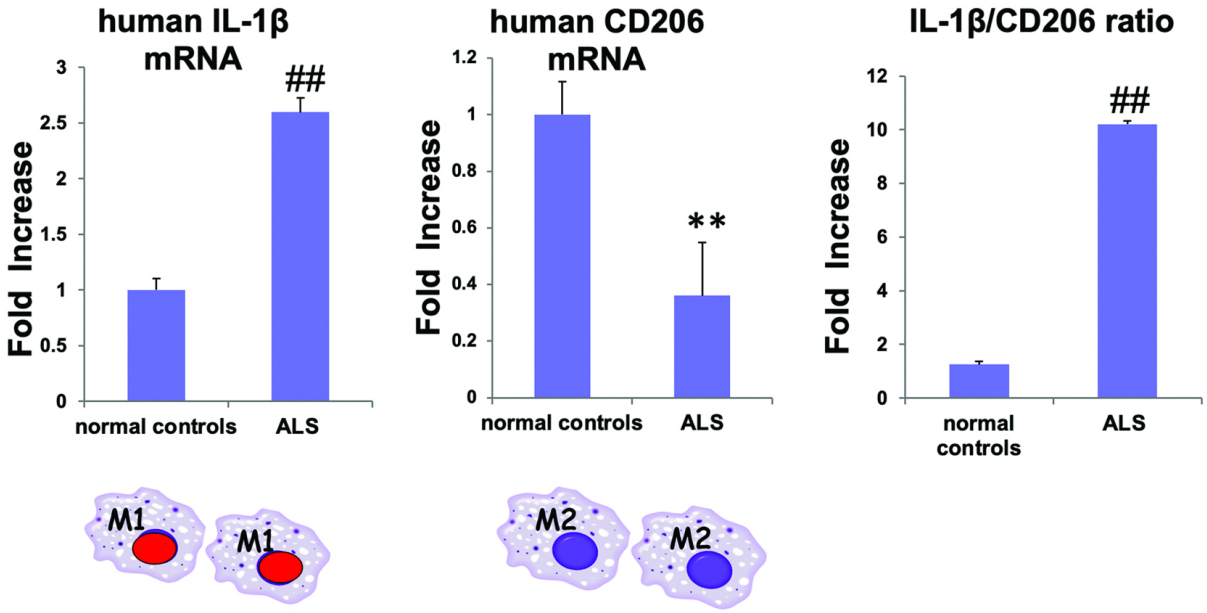

Figure 2. ALS peripheral blood monocytes are pro-inflammatory. Expression of pro-inflammatory cytokine IL-1β gene as a marker of M1 macrophages in ALS monocytes is compared to normal control monocytes in the left panel. The expression of CD206 gene as a cell-surface protein marker of M2 is compared with normal control monocytes in the middle panel. The ratio of IL-1β/CD206 gene expressions is presented in the right panel. Monocytes of ALS patients (n = 43) were verified by qPCR and normalized to β-actin and normal control monocytes (n = 22). Error bars indicate the standard error, $^{**}P < 0.01$ and $^{**}P < 0.05$. ALS: Amyotrophic lateral sclerosis.

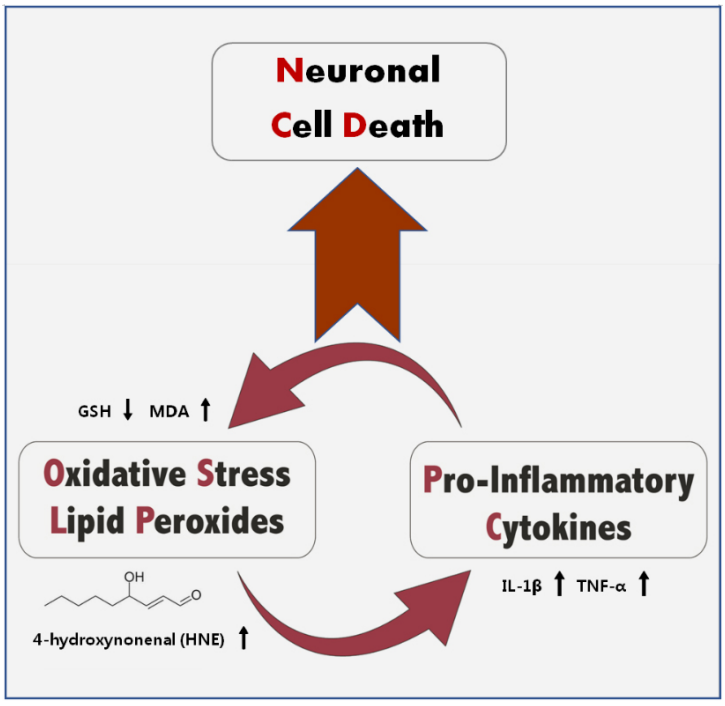

Figure 3. The significantly increased HNE lipid peroxides as well as oxidative stress could drive the activation of peripheral monocytes/macrophages enhancing the synthesis and release of pro-inflammatory cytokines. In turn, pro-inflammatory cytokines promote the production of free radicals and the synthesis of HNE. This vicious circle promotes the motor neuronal cell death in ALS including ferroptosis. IL-1β: Interleukine-1β; TNF-α: tumor necrosis factor α; GSH: glutathione; MDA: malondialdehyde; HNE: 4-hydroxynonenal; ALS: amyotrophic lateral sclerosis.

PERIPHERAL BIOMARKERS OF *INFLAMMATION*-ACUTE PHASE PROTEINS

Activation of circulating monocytes to a pro-inflammatory state induces the shedding of membrane-bound CD14 (mCD14), increasing serum levels of the acute phase protein (APP) soluble CD14 (sCD14) and enhancing the production of interleukins and TNF-α[25]. The pro-inflammatory state of macrophages was also associated with the production of the APP, C reactive protein (CRP)[26]. Elevated serum CRP levels were associated with faster disease progression in ALS patients[27]. Lipopolysaccharide binding protein (LBP) is another APP whose synthesis is increased in ALS and is enhanced as a component of the acute phase response to tissue trauma or inflammation[28]. The acute phase response accompanies chronic as well as acute inflammatory states. The APPs are synthesized and secreted from liver hepatocytes stimulated by inflammatory cytokines including IL-6, IL-1β, and TNF-α.

In ALS patients, serum levels of APPs, including sCD14, LBP, and CRP, are elevated and correlate positively with increased disease burden and faster disease progression[29]. Levels of APPs predicted survival times. In a 3-year follow-up, 72% of the patients with sCD14 levels above the Receiver Operating Characteristic cutoff values were deceased, whereas only 28% below the cutoff were deceased. Thus, the increased levels of APPs in ALS patients accurately reflect disease burden, progression rates, and survival times. The APPs were not elevated in the blood of patients with Alzheimer's Disease (AD), Parkinson's Disease (PD) or frontotemporal dementia (FTD). The increased levels of these liver-synthesized proteins confirm the concept of ALS as a widespread systemic disorder, which is distinctive from other neurodegenerative disorders such as AD, PD, or FTD. The fact that the acute phase response and the increased APPs produced by the liver are initiated by IL-6, IL-1β, and TNF-α, clearly suggests that activated pro-inflammatory macrophages, which release these cytokines, also play a key role in stimulating the increased synthesis of APPs.

BIOMARKERS OF OXIDATIVE STRESS- LIPID PEROXIDE HNE

Activation of macrophages in ALS not only enhances the synthesis and secretion of pro-inflammatory cytokines, but also increases markers of oxidative stress including superoxide anion and nitric oxide. These latter free radicals can non-enzymatically lead to lipid peroxides, specifically HNE[30]. A meta-analysis of markers of oxidative stress in ALS showed significantly increased blood levels of 8-hydroxyguanosine, malondialdehyde, and advanced oxidation protein product[31]. Levels of GSH were significantly reduced. Thus, both pro-inflammatory responses and markers of oxidative stress are increased; the pro-inflammatory responses exacerbate oxidative stress, and the oxidative stress exacerbates pro-inflammatory responses[32,33]. One of the major markers of oxidative stress in neurodegenerative diseases is HNE, which results from peroxidation of PUFAs, especially arachidonic acid. HNE promotes the formation of toxic protein adducts, which are increased in the spinal cord and ventral horn motor neurons in ALS patients[34].

HNE was significantly elevated in the cerebrospinal fluid (CSF) of sporadic ALS (sALS) patients; levels of HNE were 1.82 ± 0.15 ng/mL in 186 sALS patients compared with 0.51 ± 0.05 ng/mL in 236 patients with other neurological diagnoses[35]. Levels of HNE were considerably less increased in patients with familial ALS, PD, and AD or patients with immune or nonimmune neurological diseases. No differences were noted in sALS patients comparing those with limb *vs.* bulbar onset. 130 limb-onset sALS patients had mean CSF HNE values of 1.77 ± 0.17 ng/mL, and 56 patients with bulbar-onset sALS had levels of 1.79 ± 0.25 ng/mL. Nanomolar concentrations of free HNE or sALS.

HNE is neurotoxic in vitro. CSF samples containing equivalent HNE or HNE adducts were toxic to a motor neuron cell line in vitro. Incubation with the VSC4.1 cell line caused significant cell loss, which could be rescued by co-incubation with GSH[35]. HNE is also cytotoxic *in vivo*. Intrathecal administration of HNE to

rats increased CSF HNE and was toxic to spinal motor neurons. Total calcium was reduced in the surviving, structurally intact motor neurons, but only if GSH synthesis was concomitantly inhibited. Thus the *in vivo* toxic effects of HNE are dependent on a reduction of GSH. GSH is neuroprotective and reduced GSH level causes increased CSF HNE and enhanced motor neuron loss[36].

To determine whether oxidative stress was systemically increased in ALS, we analyzed serum as well as CSF levels of HNE using high-performance liquid chromatography and ELISA and compared them with levels in disease and normal control subjects[37]. HNE levels were significantly elevated in the sera and spinal fluid of sALS patients compared with control populations. sALS HNE serum and CSF levels were elevated above all control values [Figure 4]. CSF HNE levels were significantly increased compared with serum HNE levels in sALS patients ($P < 0.001$). As the burden of the disease increased from early to mid to late stages, HNE was increased and correlated with disease extent but not rates of progression[37]. HNE protein adducts have been reported to result from increased intraneuronal calcium and mitochondrial dysfunction, thereby supporting an important role for motor neurons in initiating the cytotoxic environment[38,39].

BIOMARKERS OF OXIDATIVE STRESS- OX LDL

Oxidative stress is an important trigger of lipid oxidation[40]; the presence of oxidized low-density lipoprotein (ox-LDL) in the serum reflects significantly increased inflammation. Lectin-like oxLDL receptor-1 (LOX-1) is the main receptor for oxLDL on macrophages, and internalizes and then degrades ox-LDL. Scavenger receptors on macrophages can also bind oxLDL. Binding of ox-LDL to LOX-1 on macrophages activates NF-κB, stimulating the production of the pro-inflammatory cytokines IL-1β and IL-18[32]. In ALS patients, serum levels of oxLDL are increased, primarily in patients with rapidly progressing disease[32] [Figure 5].

IMMUNOMODULATORY THERAPY FOR ALS

Given the evidence for neuroinflammation and oxidative stress systemically as well as within the CNS compartment, our own therapeutic efforts have been to suppress activated macrophages and microglia as well as T-effector lymphocytes that promote neuroinflammation. We have focused on regulatory T lymphocytes (Tregs), which are the $CD4^{+}CD25^{high}FOXP3^{+}$ subpopulation of T-lymphocytes that promote neuroprotection by suppressing pro-inflammatory responses. Tregs are dysfunctional in ALS; their ability to suppress the release of cytokines from pro-inflammatory myeloid macrophages and microglia is impaired, as is their ability to suppress the proliferation of T-effector lymphocytes[41]. The failure to effectively suppress central and peripheral inflammation promotes the pathogenesis of the disease, increasing the rate of disease progression and disease burden.

Our discovery of the central role of Tregs in ALS came as a result of crossing the *mSOD1* transgenic mouse model of ALS with a $Rag2^{-/-}$ transgenic mouse and a $CD4^{-/-}$ transgenic mouse. We had assumed that the offspring mSOD1/$Rag2^{-/-}$ or SOD1/$CD4^{-/-}$ double transgenic mice would live longer because we had deleted pro-inflammatory T-effector cells. However, the double transgenic mice died earlier than expected, suggesting the loss of a neuroprotective population. In these transgenic mice, pro-inflammatory macrophages and microglia were significantly increased, as were CNS pro-inflammatory cytokines. Adoptive transfer of mouse Treg cells into these doubly transgenic mice prolonged survival by 88%, and pro-inflammatory phenotypes were suppressed[2,42].

In patients with ALS, inflammation is associated with decreased numbers of circulating Tregs, and decreased expression of FoxP3, the key transcription factor in the development and function of Tregs. As a result, neuroprotective functions were diminished. The ability of circulating Tregs to suppress either

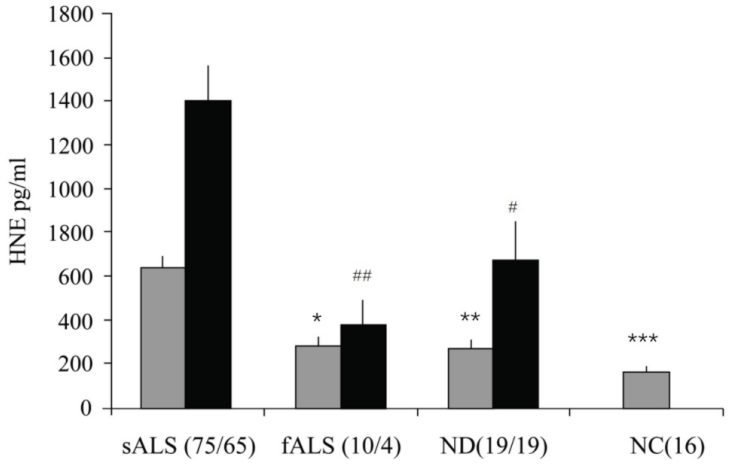

Figure 4. Serum and CSF levels of HNE in patients with sporadic ALS (sALS), familial ALS (fALS), non-ALS neurodegenerative diseases (ND) compared with normal controls (NC). Serum HNE levels in 75 patients with sALS (grey columns) were significantly increased compared to NCs ($P < 0.0001$, 10 fALS ($P < 0.05$), and 19 NDs ($P < 0.001$). CSF HNE levels (black columns) were significantly increased compared with serum HNE levels in sALS patients ($P < 0.001$). ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$, compared with sALS sera; ##$P < 0.001$, #$P < 0.05$, compared with sALS CSF (Modified from Figure 1, Ref. 37[37]). CSF: Cerebrospinal fluid; HNE: 4-hydroxynonenal.

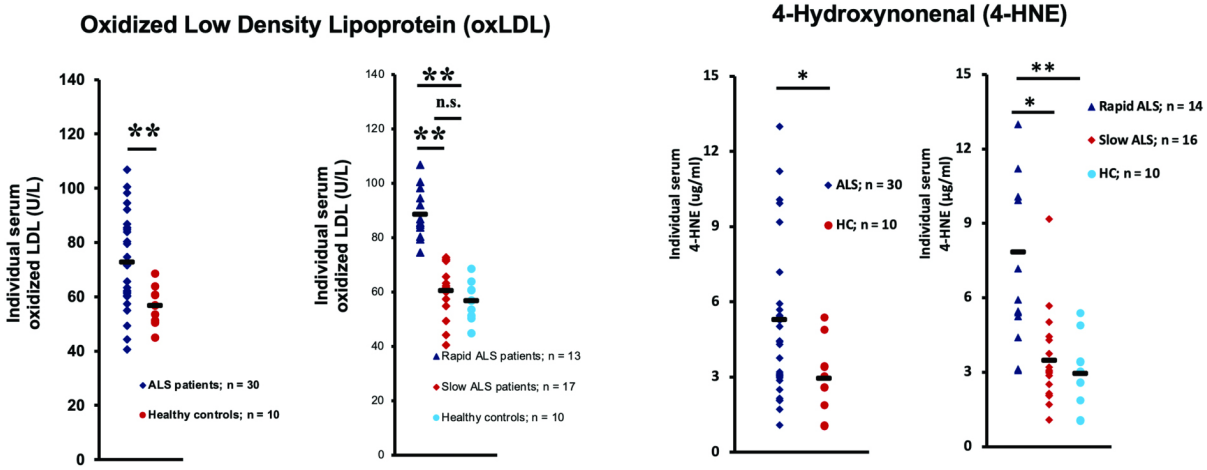

Figure 5. Serum oxLDL levels were increased in ALS compared to healthy controls. When fast progressing ALS patients were compared with slowly progressing patients, only fast progressors differed from controls. Slowly progressing patients did not differ from controls (**$P < 0.001$; n.s.= not significant). Serum HNE levels were also significantly different from controls (*$P < 0.05$), but only fast progressors differed from controls (**$P < 0.01$). Fast *vs.* slow progression was based upon changes of > 1.5 points *vs.* < 1.5 points on our AALS clinical scale[45] (Modified from Figure 1, Ref. 32[32]). ALS: Amyotrophic lateral sclerosis; HNE: 4-hydroxynonenal.

myeloid cells or T-effector cells was impaired. The more advanced the disease, the less the suppressive function. Reduced FoxP3 expression levels predict rapidly progressing disease and attenuated survival; increased FoxP3 expression levels were associated with longer survival. The impaired suppressive function also predicted shortened survival. If Tregs suppressive function was increased at baseline, three years later, 13% of ALS patients had expired, whereas decreased Treg suppressive function at baseline resulted in 35% of ALS patients having expired[43]. Thus, Treg suppressive functions are impaired in ALS, but withdrawal of Tregs from the blood of ALS patients and expansion ex vivo in the presence of IL-2 restored and enhanced their suppressive functions[44].

Autologous infusions of these expanded Tregs, together with subcutaneous IL-2 injections, formed the basis of two ALS clinical trials. In the first trial, three patients were selected to participate in an FDA-approved pilot study of autologous infusions of expanded Tregs[45]. Each of the patients was progressing at a different rate: the first patient with arm onset was progressing at an intermediate rate, the second patient with bulbar onset was progressing at a rapid rate, and the third patient was progressing at a slow rate. Regulatory T lymphocytes from all three patients had decreased ability to suppress T-effector proliferation in vitro. However, following ex vivo expansion, the in vitro suppressive function in all three patients was restored. The patient's own expanded Tregs were infused intravenously every 2 weeks for a total of four infusions, together with subcutaneous injections of IL-2 three times weekly. When the infusions stopped, even though IL-2 was continued, the patient's clinical status deteriorated. After a 4-6 months hiatus, four monthly autologous infusions again slowed clinical progression. Once again, when infusions were stopped, the clinical condition deteriorated.

In all three patients, infusions were safe and well-tolerated and slowed progression rates during early and later stages of the disease. Treg numbers and suppressive function increased after each infusion and correlated with slowing of disease progression. However, the limited duration of the clinical benefit was initially unexplained. Only in retrospect did it become apparent that the serum biomarkers of oxidative stress, HNE and oxidized LDL provided a potential explanation. These lipid peroxide biomarkers were increased prior to Treg infusions, fell with Treg infusions and slowing of disease progression, rose again as disease progression accelerated in the absence of infused Tregs, then fell again when Tregs were reinfused[32]. Thus, the fall or rise of HNE and ox-LDL levels were effectively responsive to Treg infusions and mirrored the stabilization or deterioration of the subject's clinical status.

A Phase 2A study of autologous infusions of expanded Tregs in combination with subcutaneous IL-2 injections was undertaken at Houston Methodist and Massachusetts General Hospitals[46]. The trial was planned for 12 ALS patients enrolled in a 24-week double-blind placebo-controlled trial (RT) followed by a 24-week open-label extension (OLE). In the RT portion, Treg/IL-2 treatments were safe and well-tolerated with increased Treg suppressive function in the active group. However, the COVID-19 pandemic reduced the number of ALS patients enrolled to six and precluded a meaningful statistical comparison of the efficacy of Treg infusions versus control infusions. However, the six patients plus two additional OLE-only ALS participants were able to complete the 24-week OLE; Treg/IL-2 treatments were safe and well-tolerated and Treg suppressive function and numbers were increased. Six patients showed minimal clinical progression in the OLE with the ALS Functional Rating Scale (ALSFRS) decreasing by only 2.7 points over 24 weeks, while two patients were unresponsive to Treg/IL-2 infusions with the ALSFRS decreasing by 10.5 points over 24 weeks [Figure 6]. These two rapidly progressing patients had elevated levels of two markers of peripheral inflammation (IL-17C and IL-17F) as well as a marker of oxidative stress, oxLDL. Normal levels of IL-17C and 17F, as well as oxLDL, were present in the six participants that responded to Treg/IL-2 infusions with slowed progression. The two ALS participants that were unresponsive to Treg/IL-2 infusions and progressed rapidly also had significantly increased levels of HNE, while the six responder participants had normal HNE levels [Figure 7]. Thus, in the open-label Phase 1 trial and the open-label extension of the Phase 2 trial, the biomarkers of oxidative stress were reduced, paralleling the responsiveness to therapy; unresponsiveness of Treg/IL-2 infusions to significantly elevated levels of HNE and oxLDL was associated with lack of therapeutic benefit. Levels of neurofilament light were unchanged throughout both studies and did not serve either as a prognostic or responsiveness biomarker. HNE and oxLDL not only contribute to the pathogenesis of the disease, but also serve as meaningful biomarkers of responsiveness to immunomodulatory therapy.

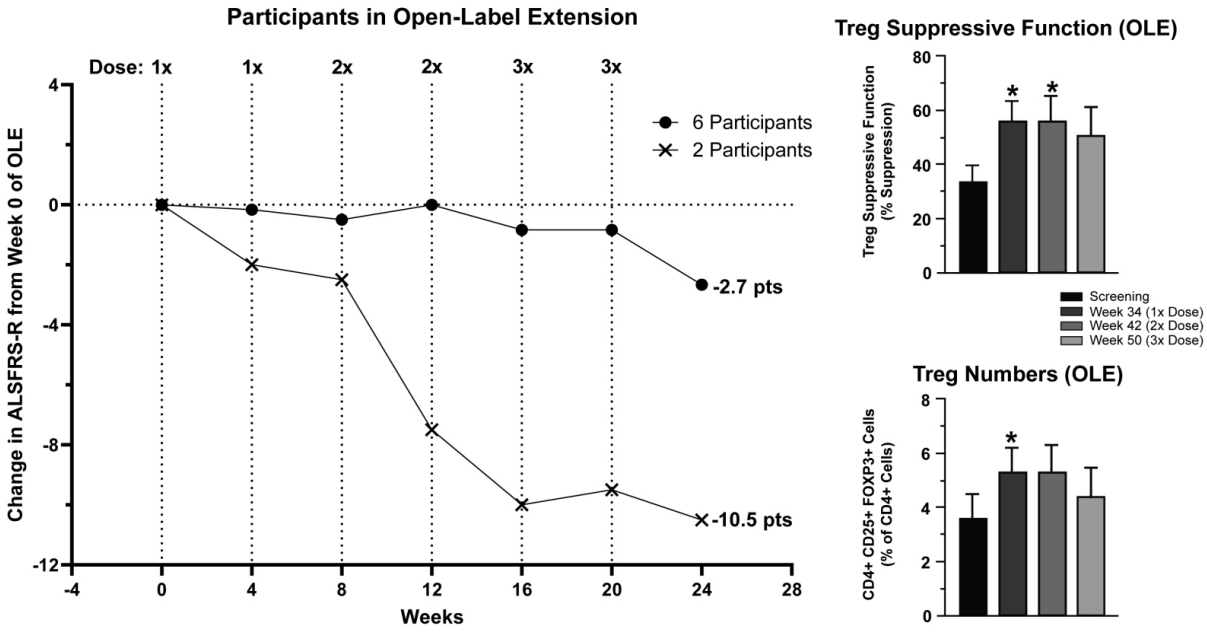

Figure 6. Treg numbers and suppressive function of Tresponder proliferation and disease progression in ALS patients in the Open Label Extension (OLE) of the Phase2A study[46]. The dose escalation was 1X, 2X, and 3X q2 months during the 24 weeks of the Open Label Period. Both Treg numbers and suppressive functions were increased (*$P < 0.05$) for at least the first 2 weeks. During the OLE the progression rate was slowed in the 6 ALS patients that appeared to have responded to the Treg/IL-2 infusions as monitored by the ALS Functional Rating Scale-Revised; whereas 2 ALS patients were unresponsive to the Treg/IL-2 infusions. ALS: Amyotrophic lateral sclerosis.

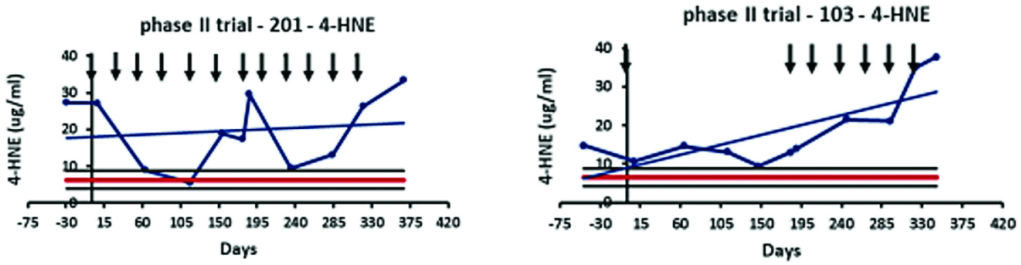

Figure 7. Serum HNE levels were evaluated throughout the Phase 2 A trial. HNE was significantly elevated in the 2 ALS patients who progressed rapidly despite escalating doses of Treg/IL-2 infusions during the Open Label Extension (OLE). The down arrows indicate dates of infusions Patient 201 (Left) had received 12 monthly Treg/IL-2 infusions while patient 103 (Right) received 6 monthly infusions only during the OLE. Both patients progressed rapidly and both had markedly increased HNE. The 6 ALS patients whose progression appeared to have slowed during the OLE had HNE levels within the normal range averaging less than 10 μg/mL. The red line indicates the mean of healthy controls and the green lines ± 1 standard deviation. ALS: Amyotrophic lateral sclerosis; HNE: 4-hydroxynonenal.

CONCLUSIONS

Systemic inflammation drives the pathogenesis of the disease in ALS. Alterations at the neuromuscular junction represent the early stages of neurodegeneration. Axon terminal mitochondria are swollen and disrupted, and intraluminal and intramitochondrial calcium are increased and promote ferroptosis. Lipid peroxides and oxidative stress are increased, monocyte/macrophages and cytokines are pro-inflammatory, and Tregs are dysfunctional. Oxidative stress promotes pro-inflammatory immune activation, and pro-inflammatory immune activation promotes oxidative stress. Peripherally activated macrophages and T lymphocytes spread from the periphery to the CNS and from the CNS back to the periphery, amplifying microglia/T lymphocyte-mediated neuroinflammation and self-propagating neurotoxicity. Lipid peroxides

not only drive the systemic inflammation, but also are biomarkers of the ongoing inflammatory cascade. Dysfunctional Tregs fail to provide neuroprotection. However, following ex vivo expansion, Treg suppressive functions are restored; autologous infusions of expanded Tregs in two small open-label studies suppressed pro-inflammatory lipid peroxides with promising clinical results. Only a large double-blind placebo-controlled clinical trial can determine whether Treg/IL-2 autologous infusions can slow disease progression in ALS patients. Our studies suggest that suppressing peripheral oxidative stress-mediated inflammation may provide disease-modifying therapy in ALS.

DECLARATIONS

Author's contribution

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

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Conflicts of interest

"No conflicts of interest influencing the representation or interpretation of reported research results". However, I do have the following disclosures-none of which are involved with this manuscript. Mitsubishi Pharma- Scientific Advisory Board; Eledon- Consultant; Implicit, Inc - Consultant; Coya Therapeutics- Scientific Advisory Board; Coya Therapeutics- Treg data licensed from Methodist Research Institute.

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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Caffeine, chocolate, and adenosine antagonism in Parkinson's disease

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is generally accepted that dopamine replacement therapy substantially improves motor symptoms; however, there is a worldwide tendency to include nutrients in treatment strategies. In the present review, caffeine and chocolate are discussed. Caffeine use seems to postpone the occurrence of PD in men, and perhaps also in women who do not take postmenopausal hormone replacement therapy. There are contradictory data concerning possible caffeine-induced improvements in PD symptoms. Given that the basic action of caffeine is the antagonism of adenosine receptors, adenosine antagonists may be a new option for treating PD patients. Furthermore, PD patients tend to have increased chocolate consumption; this may be causally related to ingredients such as phenylethylamine. Thus, nutrients such as caffeine and chocolate may play an important role in postponing and/or improving symptoms in PD.

Keywords: Parkinson's disease, caffeine, chocolate, adenosine antagonism

INTRODUCTION

Idiopathic Parkinson's disease (PD) is the second most common neurodegenerative disorder and is rapidly increasing in incidence[1]. It is generally assumed that misfolded α-synuclein is the main constituent of Lewy bodies[2] and is the major player in the etiopathogenesis of PD. Inflammation and oxidative stress also

appear to have an important role in the death of dopaminergic neurons in this disease. Furthermore, PD seems to be a spreading disease[3,4] that affects not only the dopaminergic nigrostriatal system, but also many other parts of the brain and autonomic nervous system[3]. The widespread appearance of misfolded α-synuclein causes the typical motor symptoms associated with PD - akinesia, rigidity, and tremor - and impairs the production and function of other neurotransmitters, thus leading to cognitive, psychiatric, autonomic, and other symptoms. Treatment of PD is mostly focused on repairing imbalances between the direct and indirect pathways (i.e., addressing and substituting the dopaminergic system by administering dopamine replacement therapy).

It would likely be well accepted if readily available and commonly used nutrients were able to improve the symptoms of PD or even postpone its onset. In light of this assumption, the following review focuses on our knowledge of caffeine and chocolate in the control of PD and also reviews the use of adenosine antagonists as a therapeutic approach.

FUNCTION OF CAFFEINE IN THE BRAIN

Caffeine (1,3,7-trimethylxanthine) is the most frequently used psychostimulant worldwide[5]. It is a natural alkaloid and can be found in leaves and seeds (e.g., from coffee and cacao plants), from which it can be extracted. The stimulating effects of the cacao plant were recognized by the Maya culture, which led to its cultivation as early as 1000 BC. Caffeine can cross the blood-brain barrier and exerts its biological effects mainly via the antagonism of adenosine receptors[5]. It inhibits lipid peroxidation and the formation of reactive oxygen species[6,7] and improves mitochondrial function[8]. Caffeine is metabolized in the liver; its main metabolites are paraxanthine, theobromine, and theophylline. In common with its three metabolites, caffeine can cross all biological membranes and be excreted in urine. Because of its lipophilic structure, caffeine can also cross the blood-brain barrier and elicit its effects in the brain. Its blockade of the adenosine A1, A2A, and A3 receptors in glial cells, astrocytes, oligodendrocytes, and neurons modulates the release of dopamine, serotonin, acetylcholine, γ-aminobutyric acid (GABA), adrenaline, and noradrenaline in the nucleus accumbens, hippocampus and other brain regions, including the nigrostriatal system[5,9,10]. Caffeine also inhibits phosphodiesterases and causes calcium release from intracellular storage. Moreover, at moderate doses of 3-5 cups of coffee per day, it improves sleep, learning ability, cognition, and mobility. It thus seems clear that such a substance may have beneficial effects for patients with movement disorders, depression, migraines, or dementia[7,11-14]. Nonetheless, too high a dose of caffeine causes dysphoria, restlessness, nausea, and vomiting. The lethal dose for humans seems to be 100 cups of coffee or 10 g of caffeine per day[15].

CAFFEINE AND PD RISK

There is evidence from animal models that caffeine may reduce the risk of developing PD[16]. The adenosine A2A receptor seems to play a key role in this protection in animal models of PD [e.g., the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model], and an A2A antagonist (KW6002; istradefylline) has been developed to treat patients with PD[17]. The basic mechanism of the protective effect of caffeine/adenosine antagonists in animal models is not yet fully established, but may involve the prevention of blood-brain barrier disruption in addition to neuroprotective and antioxidative effects[18].

The first evidence of a possible neuroprotective effect of caffeine against PD came from the Honolulu Heart Program, in which more than 8000 participants were followed for 30 years or more. A high intake of caffeine (> 784 mg caffeine/day) reduced the risk of developing PD by fivefold[19]. Paganini-Hill[20], who analyzed 395 patients from a retirement community in Southern California, obtained similar results. The author demonstrated that the risk of developing PD was significantly reduced in smokers, hypertensives,

alcohol consumers, and coffee drinkers. The literature at that time pointed toward a tendency for men to benefit more from coffee than women with respect to PD; thus, Ascherio *et al.* prospectively investigated the interplay between estrogen and coffee consumption[21]. They reported that caffeine reduces the risk of PD in women who do not use postmenopausal hormones, but increases the risk among hormone users. These findings suggest that women should avoid caffeine in combination with postmenopausal hormones. A study from Sweden of 415 same-sex twin pairs analyzed the influence of many lifestyle factors - such as smoking, alcohol, area of living, education, and caffeine consumption - on the risk of developing PD[22]. Only smoking was found to have a positive effect. However, a major problem with this study was that, although coffee consumption led to a reduced risk of PD, the finding was not significant; this was likely because the controls also drank relatively high amounts of coffee. In contrast to the findings of Ascherio *et al.*, a large prospective Finnish study demonstrated that caffeine consumption was associated with a lower risk of PD in both men and women[23]. Because tea also contains caffeine, it is noteworthy that a study from Singapore demonstrated positive effects of black tea - but not green tea - on risk reduction in PD[24]. In a further study, Powers *et al.* investigated the effects of a combination of coffee, smoking, and the regular intake of nonsteroidal anti-inflammatory drugs on the risk of developing PD[25]. They found a reduced risk of up to 87% for those who used all three, and their results also indicated dose-dependent effects. Similar results were obtained by Sääksjärvi *et al.*[26]. In another large prospective study involving more than 300,000 participants, caffeine consumption was once again found to be associated with a reduced risk of developing PD in both men and women[27]. A more recent study of more than 900,000 participants reported that 3 cups of coffee per day is the most beneficial for preventing PD[28]. In conclusion, it seems that coffee drinking helps to lower the risk of developing PD. A summary of these studies is provided in Table 1.

CAFFEINE AS A POSSIBLE TREATMENT FOR PATIENTS WITH PD

If caffeine is protective via adenosine receptors, it should also be determined whether it has beneficial effects in patients with established PD. In the Harvard Biomarkers Study, a longitudinal study involving 369 patients with PD - of whom 97 were *de novo* patients - and 197 healthy controls, high caffeine consumption resulted in a delayed need to start levodopa therapy[29]. Patients consumed an average of 296 mg of caffeine per day, and those who consumed less caffeine had a higher prevalence of PD and more rapid disease progression. Higher espresso consumption also correlated with improved motor function [using the Unified Parkinson's Disease Rating Scale (UPDRS)] and non-motor symptoms (using the Nonmotor-Symptoms Questionnaire). Prior to this, Altman *et al.* had reported that caffeine was associated with improved motor and non-motor symptoms in a smaller series of PD patients[30]. Simon *et al.* evaluated the rate of disease progression when creatine, another possible neuroprotective substance, was administered in addition to coffee[31]. This Phase III study involved 1741 PD patients; information about caffeine intake was available from 1549 participants. The influence of caffeine was analyzed using the UPDRS and the observation period was up to 5 years. There was no indication that caffeine had a beneficial effect on PD progression; on the contrary, caffeine combined with creatine was associated with a negative effect. In a randomized, controlled trial, patients with PD of 1-8 years of duration, Hoehn and Yahr stages I-III, and on stable symptomatic therapy were randomized to 200 mg caffeine or placebo capsules twice daily for 6-18 months. There was no improvement in either group (61 participants with placebo and 57 with caffeine) in the Movement Disorder Society UPDRS. There was a slight improvement in somnolence during the first 6 months as well as a slight increase in dyskinesia and worse cognitive testing scores in the caffeine group[32]. Thus, the same research team that observed a positive effect of caffeine (with a decrease in UPDRS of 3.2 points) in a smaller, randomized trial of PD patients who did or did not receive caffeine[33] found in this later study that caffeine was not associated with improvements in the condition of PD patients. The authors themselves stated that this difference may have been caused by different study populations. In the positive trial, patients were older and somnolent, had a longer disease duration, and were more often male. In favor of the negative study, it

Table 1. Caffeine consumption and risk of developing PD

Ross *et al.*, 2000[19]	Honolulu Heart Programme	Caffeine reduced the risk of developing PD
Paganini-Hill *et al.*, 2001[20]	Retirement Community South California	Caffeine and smoking reduced the risk of developing PD
Ascherio *et al.*, 2003[21]		Caffeine reduces the risk of PD in men and in women who do not use postmenopausal hormones
Wirdefeldt *et al.*, 2005[22]	Swedish twin study	Nicotine but not caffeine reduced the risk of developing PD
Hu *et al.*, 2007[23]	Finnish study	Both men and women show a reduced risk of developing PD
Powers *et al.*, 2008[25] Sääksjärvi *et al.*, 2008[26]		If smoking and regular intake of nonsteroidal anti-inflammatory drugs are used in combination with caffeine, the risk of developing PD can be further reduced
Liu *et al.*, 2012[27]		Caffeine reduces the risk of developing PD
Qi*et al.*, 2014[28]		Caffeine reduces the risk of developing PD

PD: Parkinson's disease.

lasted for 6 months compared with 6 weeks in the positive study. In this context, it is surprising that the patients who received caffeine had poorer performance in the Montreal Cognitive Assessment Scale; this finding is contradictory to the normal stimulant effects of caffeine on alertness and cognitive function. Thus, it remains unclear whether caffeine improves motor or/and non-motor symptoms in PD. A summary of the studies is provided in Table 2.

CAFFEINE AS A BIOMARKER FOR PD

Fujimaki *et al.* analyzed the levels of caffeine and 11 of its metabolites in the serum of 108 advanced-stage PD patients and compared them with the levels found in the serum of 31 healthy age- and sex-matched controls[34]. Independent of disease stage, total caffeine intake, or disease severity, the levels of caffeine and nine metabolites - including theophylline, theobromine, and paraxanthine - were decreased in PD patients. Caffeine levels were, on average, 25% of those in healthy controls, theophylline 41%, theobromine 50%, and paraxanthine 42%. No genetic variants in *CYP1A2* or *CYP2E1*, which encode cytochrome P450 enzymes that are primarily involved in metabolizing caffeine in humans, were identified compared with controls. Patients with dyskinesia had lower serum caffeine concentrations than those without dyskinesia. In addition, the authors were unable to detect genetic abnormalities in the gene encoding adenosine A2A receptors. Most of the patients and controls drank 1-3 cups of coffee per day and there was no difference between the two groups in the amount of coffee consumed. The findings of this study suggest that caffeine and its metabolites may be used as biomarkers for PD. A reasonable explanation for this observation may be that caffeine absorption is reduced in PD patients. Ohmichi *et al.* assessed the measurement of theophylline as a possible new biomarker in the serum of PD patients[35]. Theophylline is a major metabolite of caffeine and is advantageous because it can easily be analyzed in most hospitals using standardized immunoassay kits. In addition, theophylline levels are less markedly affected by caffeine intake. The authors measured theophylline concentration in the serum of 31 patients with PD and 33 age-matched controls. On average, PD patients had 50% less theophylline in their serum than control individuals. The only weakness of this study is that it remains uncertain whether theophylline concentration is affected by coffee consumed before blood is drawn; further studies are therefore needed. The same group developed a specific enzyme-linked immunosorbent assay system for detecting caffeine in blood[36]. In a series of 50 PD patients, 50 multiple system atrophy (MSA) patients, and 45 age-matched controls, serum caffeine concentration was significantly lower in PD patients (and, to a lesser extent, MSA patients) than in controls. In a first cohort of only 18 MSA patients, there was no significant difference between MSA patients and controls. Crotty *et al.*

Table 2. Caffeine consumption and Parkinsonian symptoms

Altmanet *al.*, 2011[30]		Caffeine improved both motor and non-motor symptoms in PD patients
Bakshiet *al.*, 2020[29]	Harvard Biomarkers Study	Caffeine use resulted in later start with levodopa. High caffeine intake reduced the progression of the disease and improved motor function
Simon *et al.*, 2015[31]	Creatine phase III study	No improvement of motor symptoms by caffeine and worsening when creatine was added
Postuma *et al.*, 2012[33]		Improvement of UPDRS part III by 3,2 points
Postuma *et al.*, 2017[32]	Café-PD	No improvement of motor symptoms in patients with PD

PD: Parkinson's disease.

studied caffeine, among other analytes, in the cerebrospinal fluid and serum of 118 patients with a pathogenic mutation in the gene encoding leucine-rich repeat kinase 2 (*LRRK2*) and PD, 115 patients with a pathogenic mutation in *LRRK2* but without PD symptoms, 70 idiopathic PD patients without *LRKK2* mutations, and 68 controls[37]. Plasma caffeine concentration was lower in patients with idiopathic or *LRRK2* -positive PD than in unaffected *LRKK2*-positive individuals, and was lower in *LRRK2*-positive PD than in idiopathic PD. It is intriguing that patients with PD and *LRKK2* mutations had lower caffeine and metabolite levels than *LRRK2*-positive carriers without motor symptoms; this was true for both the cerebrospinal fluid and plasma. Notably, the *LRRK2*-positive patients with motor symptoms drank much less caffeine than those without symptoms. Thus, it may be that *LRKK2*-positive patients without PD symptoms drank more caffeine and were protected by attenuation of LRRK2-potentiated α-synuclein pathology. These observations encourage a long-term study on caffeine intake in *LRRK2*-positive carriers. Taken together, caffeine and its metabolites are promising biomarkers for PD.

ADENOSINE ANTAGONISM IN PD

Adenosine is an important neurotransmitter for neuronal maturation/development, sleep and arousal, cognition and memory, and control of respiration[14]. A detailed description of the distribution, biochemistry, and functions of striatal adenosine A2A receptors can be found in Svenningsson *et al.*[38]. In short, the main actions of caffeine in the brain are in adenosine receptor antagonism, intracellular Ca^{2+} release, and GABA receptor modulation[14]. Adenosine receptors can be found in the striatum, globus pallidus, nucleus accumbens, and olfactory bulb. For PD, adenosine A2A receptors in the medium spiny neurons of the striatum are almost exclusively relevant. It thus makes sense to speculate that, similar to caffeine, drugs that antagonize adenosine receptors may be beneficial in PD. In this context, a positron emission tomography study by Ishibashi *et al.* is important; these authors demonstrated a significant occupancy of adenosine A2A receptors after participants drank a cup of coffee (equivalent to 100 mg caffeine)[39]. To date, there is only one licensed A2A receptor antagonist medication: istradefylline. This substance received approval in Japan in 2013 and in the USA in 2019; however, approval was recently denied by the European Medicines Agency. Istradefylline has been investigated in a large Phase II and Phase III clinical research program of about nine trials. On average, a gain of about 45-60 min of ON state and a decrease of about 45-60 min of OFF state have been identified. The most common side effect was dyskinesia, which was able to be overcome by adjusting levodopa dose[40].

CHOCOLATE AND PD

Chocolate, particularly dark chocolate, also contains caffeine. Specifically, 100 g of dark chocolate contains 43 mg of caffeine[41]. Cacao-containing chocolate is highly valued worldwide for its taste and smell, as well as for its psychoactive stimulation. Approximately 20 years ago, I noticed that the bedside cupboard of a

patient of mine - an in-patient who had traveled a long distance - was full of bars of chocolate. He told me that he had not been certain that we could provide him with his favorite chocolate, which improved his PD symptoms. On the basis of this observation, we performed a survey among our PD patients[41] and revealed a significantly higher intake of chocolate in patients than that of their caregivers and partners; this preference for chocolate was independent of the presence or absence of depression. Furthermore, the consumption of other sweets was similar between PD patients and their caregivers and partners. Caffeine may be an underlying reason for this behavior, or perhaps energy production from glucose. The amine phenylethylamine, which can penetrate the blood-brain barrier, may also be responsible for our finding[42], or tryptophane, which is a precursor of serotonin. We favored the idea that phenylethylamine may be the reason for the high intake of chocolate and performed a study in which we compared phenylethylamine content in the blood of patients with PD who had eaten either dark or white chocolate[42]. We tested the effects of 200 g of chocolate containing 80% cacao on the UPDRS motor score at 1 and 3 h in 26 subjects with moderate, non-fluctuating PD. The investigation was a mono-center, single-dose, investigator-blinded cross-over study using cacao-free white chocolate as the placebo. At 1 h after chocolate intake, mean UPDRS motor scores were mildly decreased compared with baseline in both treatment groups; however, only the dark chocolate results were significant [-1.3 (95% confidence interval [CI] 0.18-2.52, repeated measures analysis of variance $F = 4.783$, $P = 0.013$, Bonferroni $P = 0.021$)]. A 2 × 2 cross-over analysis revealed no significant differences between the two treatments [-0.54 ± 0.47 (95%CI: -1.50-0.42), $P = 0.258$]. Similar results were obtained 3 h after intake. Furthermore, β-phenylethylamine blood levels were unaltered. In summary, dark chocolate did not show significant improvements over white (cacao-free) chocolate in terms of PD motor function.

CONCLUSION

Caffeine and its metabolites play an important role in brain function. Caffeine may be neuroprotective against PD and slow the occurrence of this neurodegenerative disease. There are many more studies suggesting that coffee drinking lowers the risk of PD than those that suggest the opposite. However, the existing data on whether coffee, tea, or chocolate intake may improve symptoms in patients with PD are much more ambiguous. Given that caffeine acts mostly via adenosine receptors in many brain regions, further studies with new adenosine antagonists are needed.

DECLARATIONS

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Author's contribution

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Prof. Reichmann H was invited by EISAI to give lectures on the function of the adenosine antagonist *istradefylline*.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

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2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

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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.

2.3.2.5 Conclusion

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

2.3.3 Back Matter

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Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
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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8.1.1 Initial manuscript check

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