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YOUNG PERSPECTIVES FOR OLD DISEASES

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Young Perspectives for Old Diseases

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FOREWORD

Human neurodegenerative diseases rank amongst the most pressing subjects of modern Biomedical Science. Despite years of abundant investment, and a large body of knowledge generated by many top laboratories worldwide, little advance has been made towards the cure of diseases such as, for example, Alzheimer's, Parkinson's or ALS.

Experienced investigators have now battled for decades to find new leads to effective treatments. Legions of young students have joined their teams. Over the years, the latter have started independent work, building upon both existing knowledge as well as their unbiased views of both the pathogenesis and possible new therapeutic targets.

In his delightful book Advice to a young scientist, Sir Peter Medawar pointed out that *'The old-fashioned remedy for hubris was a smart blow on the head with an inflated pig's bladder — and this is in the spirit of the rebuke that may have to be administered before the young scientist injures himself in the opinions of those who would otherwise like him and wish him well'.* Such warning notwithstanding, it is equally wise to listen to what young scientists have to say. Not only many of them are perfectly aware of their standings, but also free of the partisanship we, scientists, tend to accumulate as our careers progress.

The seventeen young scientists who wrote this book got their Ph.D. degreees less than twenty, and eleven of them less than ten years ago. They are building their careers upon significant contributions to the literature on neurodegeneration. Both through my own personal collaboration with some of them, as well as by reading their chapters, I believe they should be exempt from the Medawar remedy. It is worth looking into their personal perspectives on subjects that range from basic cellular and molecular mechanisms to novel therapeutic targets and strategies to cope with neurodegenerative disease. Both young and old scientists have much to gain.

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PREFACE

Human neurodegenerative disorders produce devastating symptoms that enormously affect both patients and their relatives. The disease pathologies share specific features, such as late onset, accumulation of misfolded proteins that form aggregates in the brain and progressive degeneration of specific neuronal populations. Patients may suffer from behavioral and physical disorders, as well as severe cognitive impairments. The incidence of neurodegenerative diseases has dramatically increased with the aging of the population and although neurodegenerative diseases represent a major threat for public health, few effective treatments are currently available.

In order to discover drugs to prevent and treat neurodegenerative diseases, it is imperative to decipher the genetic and molecular mechanisms that underlie the onset and development of these disorders. Despite many years of research, scientists have not yet succeeded in understanding the precise mechanisms that lead to neurodegeneration. A number of important questions remain to be answered: how do disease-associated proteins cause neuronal dysfunction and death; what underlies cell type-specific degeneration; are these diseases age-dependent; are these gender-specific; are there common pathogenic mechanisms that underlie different neurodegenerative disorders; and how can these diseases be prevented and treated?

In this book we will review the most recent findings on scientific research that explored mechanisms and pathways for the diseases. We further explore how the current knowledge is being applied to improve the life of neurodegenerative disease patients.

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Section 1: Introduction

CHAPTER 1

The Role of Molecular and Cellular Biology in Neurodegenerative Diseases – Learning from Cancer Therapies

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Abstract: Increasing knowledge in molecular and cellular biology along with improved imaging technology and surgical instruments has resulted in huge advances in cancer therapy. The understanding of several cellular signaling pathways has laid the solid foundation for the development of targeted chemotherapy, which in turn, has played critical roles in increasing the survival rates of many types of cancer during the last decade. As a consequence, molecular diagnostic tests have emerged as an important step in order to plan the most appropriate treatment strategies for each case. In this chapter, we will describe classical examples of targeted cancer therapies and illustrate how similar approaches could benefit the treatment of yet incurable neurodegenerative diseases.

Keywords: DNA, genome, mutations, polymorphism, risk factors, biomarker, targeted therapy, drug discovery, prevention, early intervention, cancer, personalized medicine, molecular diagnosis, molecular and cellular biology, neurodegenerative diseases, amyloid precursor protein, α-synuclein, apolipoprotein E, Aβ-peptide clearance, Alzheimer´s disease, Parkinson´s disease.

1.1. PERSONALIZED MEDICINE

From the simple transportation of oxygen molecules to profound chemical modifications of macronutrients, every chemical and biological process that occurs in our body requires highly coordinated activities of many molecules. Unusual modifications of these molecules, whether derived from genetic factors or not, can lead to detrimental effects on health.

The majority of biologically active molecules are endogenously synthesized according to the genetic information contained in the deoxyribonucleic acid (DNA), a linear polymer composed of 4 types of nucleotide: deoxyadenosine (A),

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deoxyguanosine (G), deoxytimidine (T) and deoxycitidine (C). These nucleotides essentially differ by their nitrogenous bases, which are often used as a unit of measurement to indicate the size of DNA fragments.

The whole human genome contains approximately 3 billion base pairs [1]. Within the genome, each DNA segment that contains a fundamental sequence of nucleotides to codify a biologically functional product is called a gene. In most cases, these products are proteins, but other molecules, such as RNA (ribonucleic acid), can also be a final product of a gene. Most parts of the human genome are noncoding sequences with unclear functions, and all known human genes (roughly 30,000) are comprised of only 1% of the genome [2, 3].

Although there is a huge range of phenotypic diversity between human beings, 99.9% of the genome of one person is identical to the genome of another [1]. This means that all human genetic diversity is comprised only in 0.1% of the genome. Most commonly described genetic variations consist of differences in a single nucleotide sequence and copy number variations of a particular DNA segment resulting in chromosomal structural changes [4-6]. By definition, a difference that occurs in more than 1% of any particular population is denominated as a polymorphism, while rarer alterations are considered mutations.

To date, more than 3 million single nucleotide polymorphisms (SNPs) have been genotyped [5, 7, 8]. Most of them are localized in noncoding sequence and do not influence any known phenotype. However, certain SNPs can alter the amino acid sequence of a protein or affect gene expression control, contributing to phenotypic variability in many aspects, such as metabolism, susceptibility to diseases and response to treatments [9, 10].

In addition to SNPs, deletion, insertion, translocation and inversion of DNA fragments can occur during DNA replication and recombination. These types of alterations generate different copy numbers of particular segments in the genome of each individual, which are denominated as copy number variants (CNV) or copy number polymorphisms (CNP) [5, 6]. Although the number of CNVs identified is much smaller than the number of SNPs, the former encompasses a larger content of human genome because each CNV evolves from a few kilobases

to mega bases [6, 11, 12]. Unlike SNPs, many CNVs are located within or in flaking segments of functional sequences, suggesting that CNVs have a more significant contribution to the phenotypic variation [13].

Since the complete sequencing of the human genome, extraordinary efforts and resources have been invested to unveil information contained in the genome and to understand the genetic component of illnesses. However, the cause and cure of many diseases are still obscure. The main difficulty is that many diseases originate from multiple causes, including both genetic and environmental factors. This means that patients diagnosed with the same disease do not necessarily have the same set of causes and general pathological aspects of diseases may not explain the individual differences in the onset of symptoms, progression of the diseases and treatment outcomes. Several milestone studies have helped define molecular pathways of many diseases, showing that indeed, completely unrelated alterations can result in a similar illness. A prime example of this aspect is discussed in Chapter 13, showing that the peripheral neuropathy called Charcot-Marie-Tooth disease may be caused from mutations in more than 50 different genes. However, the phenotypic alterations in the patients are so similar that all the conditions are diagnostically grouped as one illness.

These additional molecular aspects of diseases can help to more precisely diagnose and stratify the diseases into several subgroups until each individual case is treated as a unique case: this is the main idea of personalized medicine. Personalized medicine is clinical practice based on a unique set of clinical, genetic and environmental information of an individual [14]. This type of disease management will help plan the most appropriate treatment for each case, and because the medical decisions will be made based on precise individual information, the patients are less likely to experience negative side effects and have a higher probability of the desired outcomes.

Other important concepts of personalized medicine are prevention and early intervention of diseases. Many diseases occur due to multiple causes, and each one alone may not be sufficient to develop the disease. However, a person who carries a feature associated to a specific disease has a higher risk or susceptibility to develop that disease. Thus, this person could be advised to avoid additional risk

factors or to take preemptive measures. Also, regular monitoring of health conditions can allow for the early identification of diseases and more time for intervention, which will always result in a better outcome and minimize detrimental effects of diseases.

Any genes or molecules that can specifically indicate the state of health conditions are denominated biomarkers. Biomarkers can help predict risks for disease, diagnoses, plan treatment strategies and monitor treatment responses. Current genomic medicine has validated several biomarkers for diagnosis and treatment decision of many diseases such as cancer, cardiovascular disease, neurodegenerative diseases, and neuropsychiatric disorders, among others (Table **1**). Although the health condition of an individual should not be examined only by biomarkers, quantitative or qualitative analysis of these molecules can greatly enhance the precision of clinical practice.

Table 1: Examples of biomarkers and their use in clinical practice

Biomarker	Description
HLA-B*5701	Presence of HLA-B*5701 allele is strongly associated with the hypersensitivity reaction to **abacavir**, a drug used to treat AIDS. Genetic tests prior to treatment can prevent adverse side effects [15].
HLA-B*1502	Presence of HLA-B*1502 allele is strongly associated with the development of Stevens Johnson syndrome and its related diseases in patients treated with **carbamazepin**, an anti-convulsant and mood stabilizing drug. Genetic tests prior to treatment can prevent adverse side effects [16].
CYP subfamilies	SNPs of this gene can influence the kinetic of metabolism of several drugs, including **clopidogrel**, an anti-platelet treatment [17].
UGT1A1	UGT1A1*28 polymorphism is related to toxicity of **irinotecan**, a topoisomerase inhibitor used for metastatic colorectal cancer [18].
EGFR and KRAS	**Panitumumab**, a monoclonal antibody to EGFR, is used as an adjuvant treatment of colorectal cancer with high expression of EGFR. The drug is efficacious only for patients that do not carry mutations in the KRAS gene [19].
BRAF	Metastatic melanoma with substitution of glutamic acid for valine at codon 600 of BRAF is eligible for treatment with **vemurafenib** [20].
CD30	**Brentuximab vedotin**, anti-CD30 conjugated with a cytotoxic drug (monomethylauristatin E) is used to treat CD30-positive hematological malignancy [21].
CFTR	Cystic fibrosis is a genetic disorder caused by mutations in the CFTR gene that codify for an ion channel. **Ivacaftor** is a G551D mutation-specific drug for cystic fibrosis [22].
Factor V	R506Q mutation in Factor V gene (also known as Factor V Leiden) is a risk factor for thromboembolism [23].

1.2. PROGRESS IN CANCER TREATMENT

The investigation of chemicals for systemic cancer treatment started in the early 20th century. Nitrogen mustard was the first chemical that induced remission in a group of lymphoma patients [24]. However, it turned out to be temporary and incomplete. Likewise, other drugs developed in the same period showed only tantalizing results with short extension of survival [25, 26]. Moreover, most of these chemicals had general mechanisms of action, affecting primarily the DNA metabolism. This lack of specificity contributed to severe side effects and treatment failure [27]. Thus, prior to the 1970s, the removal of a primary tumor with generous amounts of surrounding normal tissue, such as in a mastectomy, was the preferred method of treatment. For inoperable patients, radiotherapy was an option with an extremely low chance for cure or 5-year survival. These methods were also very inefficient to defeat metastatic cancer and usually ended at patient death [28]. However, after the 1970s, combinatorial drugs were used as an adjuvant systemic treatment following surgery or radiotherapy to control metastatic diseases or remaining cancerous cells and produced better results [29, 30]. A combination of drugs that have complementary mechanisms allowed the use of low doses of each chemical, reducing side effects [27, 31].

Even with scarce knowledge of cancer biology, it was evident that prevention and early intervention are the best strategies for cancer management [32]. As a result, since the 1970s, cancer research has been dramatically accelerated not only to develop more efficacious drugs and more precise surgical instruments, but also to improve imaging techniques and other diagnostic methods for early tumor detection. Also, campaigns for screening tests have greatly contributed for lowering cancer mortality [28, 33].

While cancer research continued its rapid advancement, attention shifted from the search for environmental carcinogenic chemicals to the investigation of intrinsic genetic alterations and signaling pathways that occurred in tumors [34-36]. These advances helped incorporate new concepts in cancer definition. Today, cancer can be defined as an uncontrolled cell growth accompanied by genetic instability that causes progressive genetic alterations [35-37]. In general, these alterations include gain-of-function of oncogenes and loss-of-function of tumor suppressors. The

discovery of oncogenes and tumor suppressors and their involvement in cell cycling and division was the foundation for targeted therapy, *i.e.* drugs that stimulate or inhibit the activity of a specific molecule [33].

The first targeted drug was imatinib, an inhibitor of the tyrosine kinase activity of the oncogene BCR-ABL [38]. The fusion protein BCR-ABL is originated by mutual translocation between chromosome 9 and 22, and it is found in 95% of patients with chronic myeloid leukemia. The first report on CML treatment with imatinib was promising, with 80% of patients reaching a 5-year survival [39]. However, it was intriguing why a small subset of patients did not response to imatinib, even though they produced the fusion protein. Even worse, later on, it was revealed that patients presented resistance to imatinib after long-term treatment. These questions were answered with genetic studies showing that patients treated with imatinib acquired other mutations, mostly point mutations that increase BCR-ABL phosphorylation [40, 41]. To overcome this secondary resistance, also known as acquired resistance, new drugs have been developed. Dasatinib and nilotinib are more potent inhibitors of tyrosine kinases and used to treat CML with resistance or intolerance to imatinib [42, 43]. This is an example where personalized medicine can be practiced, submitting patients to genetic testing to check for the presence of the fusion protein prior to treatment with imatinib or other related drugs and monitor during treatment for the presence of other genetic alterations. Indeed, several studies point out the importance of standardizing methods of mutation screening and defining consensus on when to screen [44, 45].

Like imatinib, there are several drugs that require prior genetic testing for application. For example, trastuzumab is a monoclonal antibody indicated for the treatment of breast cancer with HER2 gene amplification and/or overexpression, which can be detected by fluorescence *in situ* hybridization or immunohistochemistry [46]. Her2 signaling pathway cross-talks with IGF-1 and PI3K cascade, and patients that carry genetic alterations that affect these signaling molecules would not be eligible for treatment with trastuzumab [47]. Overexpression of the EGF family also decreases the efficiency of trastuzumab. Because of these intrinsic or acquired mutations that affect the efficiency of the drug, a lower percentage of patients respond to this drug [48]. Thus, more

complex genetic tests need to be performed to select eligible patients for treatment with trastuzumab. Today, several drugs have been developed to be efficient in specific cases but not in others (See examples in Table **1**). Thus, analysis of the individual genetic component has become a frequent practice in current cancer treatment to propose the most appropriate treatment.

The knowledge of molecular and cellular aspects of cancer advances rapidly, and contributes for the stratification of cancer into several smaller subgroups. However, the development of new drugs for each case occurs at a relatively slow rate. Secondary or acquired mutations are also current problems, which require more thorough genetic testing and constant devolvement of new drugs. However, a significant portion of cancer cases will become a manageable chronic disease.

1.3. POSSIBLE TARGETS FOR PERSONALIZED TREATMENT OF NEURODEGENERATIVE DISEASES

The advance in the management of several diseases and perception of the importance of a healthy lifestyle by modern society are contributing to global aging. Aging, in turn, is a well-established risk factor for several diseases such as cancer, cardiovascular diseases and neurodegenerative diseases [49-51]. Particularly, neurodegenerative diseases are estimated to increase three-fold by 2050, resulting in a huge economic burden and emotional challenge to patients and their families. In contrast to this problematic scenario, the effective strategy for diagnosis and treatment of neurodegenerative diseases is still lacking and requires urgent improvement.

Several problems make a precise diagnose difficult. First, we can evaluate the description of physiopathological characteristics of the most common neurodegenerative diseases. Memory loss, ataxia and sleep disorders are very common symptoms of various types of neurodegenerative diseases. Although careful analysis of these symptoms, including their onset and progress, can help predict the type of disease, the probability of misdiagnosis has been high [52, 53]. Thus, such broad definition and overlap of symptoms between diseases makes it difficult to correctly diagnose neurodegenerative diseases.

It is possible today to analyze the functional anatomy of the brain, such as cortical thickness and hippocampal activity, with the improvements of some imaging techniques [54, 55]. Clinical studies have demonstrated that reduction of cortical thickness or hippocampal hyperactivation and aberrant synaptic functions have been associated with a higher risk for Alzheimer's disease [56, 57]. However, reduction of cortical thickness can be observed in normal aging, and since each individual presents different thickness, it is difficult to propose a cutoff value for predictive risk. Moreover, different ethnic groups and lifestyle can influence the brain anatomy [58]. For example, elderly people subjected to physical exercise or memory training show increased cortical thickness when compared to a sedentary population [59, 60]. Hyperactivation of hippocampus can also be observed in patients with other diseases such as schizophrenia [61]. More recently, dyes that react with protein aggregates have also been proposed as a diagnostic method [62]. However, considering that many degenerative diseases present protein aggregates, the specificity of the dye has to be carefully investigated.

If the diagnostic methods for neurodegenerative diseases are poor, the therapy is even more chaotic. Currently available treatments aim to relieve the symptoms. For example, in AD, acetylcholinesterase inhibitors or memantine, an NMDA antagonist, are used to improve cognitive function and psychotic drugs in attempt to control mood disorders [63]. Levodopa, a dopamine agonist classically used in PD treatment, aims to overcome dopaminergic neuronal death [64]. However, these treatments do not have disease reversing effects and chronic treatment causes severe side effects, such as dyskinesia, hallucination, nausea, vomiting and bradyarrhythmias (for additional information see Chapter 2). Thus, if we make an analogy with cancer, the management of neurodegenerative diseases is at least 10 years behind cancer treatment, with diagnosis based on broad pathophysiological aspects and symptom relieving treatments with severe side effects [65].

The significant progress seen in cancer treatment has been achieved with multidisciplinary approaches with focus on molecular and cellular mechanisms. This means that revealing the molecular and cellular pathways involved in neurodegeneration is an important step for development of biomarkers and drug targets. Also, prevention and early diagnosis have been key elements for successful treatment of cancer. In neurodegenerative diseases, prevention might

be more critical, because regeneration or repopulation of dead neurons is a more challenging task than killing abnormally dividing cancer cells. Thus, the development of screening tests that can be easily included in a health checkup is essential. These screening tests can be affordable imaging techniques, such as mammography for breast cancer, or non-invasive molecular tests.

In this aspect, we already know subset of mutations that are causative of neurodegenerative diseases, *i.e.*, the presence of certain mutations indicate an extremely high risk to develop the disease. For example, populations that carry mutations that affect post-transcriptional modifications or expression level of the Amyloid Precursor Protein (APP), Presenilin-1 and Presenilin-2 will develop Alzheimer disease [66-68]. Likewise, mutations in α-synuclein (*SNCA*) and parkin (*PARK2*) are causative for Parkinson's disease, mutations in PrP^C (*PRNP*) cause spongiform encephalopathy and expansion of glutamine sequence in huntingtin (*HTT*) is responsible for Huntington's disease [69-73]. In general, carriers of these types of mutations present early-onset diseases.

Although hereditary neurodegenerative diseases make up only a small percentage of patients because they confer extremely high risk for the disease, well designed molecular tests for these mutations and application to appropriate populations can help identify the diseases early, allowing more time for intervention. Nonetheless, the major limitation is still the lack of effective treatment for these particular cases. Thus, even with early identification of the risk factor, at least at the present time, the patient would not get significant benefit and more dynamic research on drug development is urgently required.

Beyond these causative mutations, there are several polymorphisms that increase the risk for diseases. A well established example involves 3 variants of cysteine contents in Apolipoprotein E (*APOE*): apoE2 contains 2 cysteines, apoE3, 1 cysteine, apoE4, 0 cysteine. Studies have demonstrated that 2 copies of apoE4 alleles increase the risk for late-onset AD up to 60% [74]. The replacement of cysteine at residue 112 of apoE3 by arginine in apoE4 appears to influence secondary structure and function of these isoforms [75, 76]. In addition, other genes such as angiotensin-converting enzyme (*ACE*), *CD33*, Membrane-spanning 4-domains, subfamily A, member 6A (*MS4A6A*), Glutathione S-transferase

omega-1 and 2 genes have been also associated with increased risk for AD [77-79]. However, none of them represents as high of a risk factor as the apoE4 allele.

An increasing number of genome-wide association studies have been published during the last decade in order to find risk factors for diverse neurodegenerative disorders. Given the broad etiologic diversity of these diseases, searching for genetic risk factors is a necessary effort. These studies can help identify still unrevealed genetic alterations related to neurological disorder and improve the diagnosis of these diseases [80]. However, caution should be taken in order to not overestimate the importance of a growing list of genetic risk factors. When analyzing these studies, factors such as sample size, magnitude of risks and reproducibility of the results in other populations should be considered. Moreover, the functional aspects of each genetic alteration should be investigated with the same importance, as they can provide information for identification of the best target molecule for treatment of the disease.

Indeed, there are several mechanistic studies that investigate the involvement of known biomarkers in neurodegeneration. One of the hallmarks for AD is the accumulation of amyloid plaque, Aβ-peptide being the main component. The formation of the insoluble Aβ-peptide oligomer depends on the post-translational cleavage of APP, a glycosylated transmembrane protein with a single membrane-spanning region. APP is cleaved by α–, β– and γ- secretase. Sequential cleavage by α- and γ- secretase generate a soluble peptide, while cleavage with β- and γ-secretase produce the amyloidogenic peptide [81]. Because the amyloid plaque is the cardinal marker of AD and is influenced by genetic factors, the APP processing and Aβ clearance are current targets of AD therapy. Indeed, several γ-secretase inhibitors and monoclonal antibodies against β amyloid showed promising results in clinical studies [82-84]. However, failure of clinical trials of a γ-secretase inhibitor was recently reported. Although the inhibitor succeeded in decreasing Aβ levels up to 50%, it also caused severe side effects in the liver by mechanisms unrelated to APP processing [85]. Thus, other derivative chemicals or directed drug delivery can be tested as an alternative approach.

As mentioned above, apoE4 is a strong risk factor for AD. Although the detailed mechanism of Aβ clearance is still unclear, it appears to be dependent of apoE

isoforms, apoE2 and apoE3 being more efficient in clearance [86, 87]. Thus, compounds that can assist the correction of apoE4 structure to apoE3-like structure can also be investigated as a potential drug for AD [88].

Tau is a cytoskeletal protein that functions to stabilize microtubules. Hyperphosphorylation and aggregation of tau is another hallmark of AD [89]. The toxicity of tau aggregates is related to loss of function, affecting neurotransmission. Tau aggregation and mislocalization appear to be primed by Aβ oligomers [62, 90]. GSK-3 is one of the enzymes that phosphorylate tau and clinical trials using GSK-3 inhibitors are currently ongoing [68, 91]. However, given that GSK-3 has many substrates, significant side effects are predictable. In this case, drugs that inhibit tau aggregation such as phenothiazine methylene blue or methylene blue can be a better option [92, 93]. Clinical trial results will eventually reveal the effectiveness of these drugs.

The aggregation of α-synuclein is a canonical pathological marker for PD. α-synuclein is mostly located in presynaptic terminals and is involved in neurotransmitter release control [94]. Abnormal accumulation of α-synuclein aggregates can lead to synaptic dysfunctions [95]. Thus, maintenance of normal functions and removal of toxic aggregates can be one of the target pathways for PD. Immunotherapy, where T cells are activated with short peptides to produce antibody against synuclein aggregates has been shown as a disease modifying treatment and the first clinical trial is in progress [96, 97].

Protein aggregates can elicit inflammatory responses and increase mitochondrial permeability, leading to oxidative stress, a common phenomenon observed in many diseases [98]. Oxidative stress refers to an alteration in the balance between oxidative metabolite and enzymes that eliminate these oxidative metabolites. Thus, the enzymes that participate in these metabolic pathways or anti-oxidant enzymes can be potential candidates for targeted therapy of neurodegenerative diseases. During stress oxidative conditions, a transcription factor Nrf2 translocates into the nucleus, binds to cis-acting antioxidant response regulatory element (ARE) and controls the expression of antioxidant genes such as heme oxygenase-1 and glutathione S-transferase α4 [99]. Dimethyl fumarate and its primary metabolite monomethyl fumarate and other antioxidant compounds can

induce the expression of Nrf2, protecting against oxidative stress [100, 101]. Thus, Nrf2 is a patented promising target for treatment of neurodegenerative diseases [102].

Non-pharmacological adjuvant treatments can also be interesting options. A higher education level and occupational activities are well known protective factors for AD [103]. Physical exercise increases neurotrophic factors, such as BDNF, inducing neurogenesis and memory improvement [104, 105], while stress has adverse effects on neuronal survival and synaptic plasticity [106, 107]. It has been demonstrated that stress hormone and glucocorticoids, induce abnormal hyperphosphrylation of tau [108]. Also, sleep deprivation increases Aβ-peptide and Aβ plaque, which can be reproduced by infusion of orexin, a neuropeptide that regulates arousal [109]. Thus, a healthy lifestyle can be a simple and non-invasive strategy to reduce the risk for neurodegenerative disease.

FINAL CONSIDERATIONS

In this chapter, a subset of molecular targets for some neurodegenerative diseases that are currently under clinical trials was presented. Although the cure for neurodegenerative diseases has been extensively studied, an effective drug has not been validated, while the list of risk factors and biomarkers continues to grow rapidly. To effectively translate the genome-wide studies into applicable clinical trials, multidisciplinary research needs to be performed more actively with focus on functional genomic studies.

Another important thing to consider is the re-evaluation of failed clinical trials. In a clinical trial for trastuzumab, only a small percentage of patients benefited from the treatment, thus was considered a failed trial. However, there was a clear correlation between HER2 overexpression and responsiveness to treatment, suggesting that trastuzumab, in fact, is effective to treat a particular subpopulation of breast cancer with well defined genetic alterations. In general, clinical studies for neurodegenerative diseases are performed without a thorough selection of patients. Indeed, bapineuzumab, a monoclonal antibody that specifically targets β-amyloid, was considered failed in a phase 2 clinical trial due to a treatment-related brain image abnormality. However, further retrospective analysis demonstrated

that the imaging abnormality was correlated with apoE4 isoform and high dose [110]. Thus, failed drugs in previous clinical trials could be re-evaluated upon a stricter patient selection supported by biomarkers and genetic tests.

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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CHAPTER 2

Current Pharmacological and Non-Pharmacological Therapies for Neurodegenerative Diseases

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Abstract: Neurodegenerative disorders are an important cause of mortality and morbidity in the elderly. The most common neurodegenerative diseases are Alzheimer's disease and Parkinson's disease. Lewy body dementia is considered the third most frequent. Much less common are frontotemporal dementia, Huntington's disease, amyotrophic lateral sclerosis, progressive supranuclear palsy, spinocerebellar ataxias, Pick disease and prion disease. There is no therapy that is capable to avoid the progression of these disorders. Current pharmacological therapies offer symptomatic benefits with very little impact, if any, in modifying the course of these diseases. Anticholinesterase drugs are the most frequently used to treat Alzheimer's disease. Disease-modifying treatments for Alzheimer's disease are being developed. Levodopa is the most effective pharmacological treatment for Parkinson's disease but in long-term benefit declines. For this reason, association between levodopa and other forms of treatment is the best approach. There is no approved pharmacological treatment for most other forms of neurodegenerative diseases except for amyotrophic lateral sclerosis, Huntington disease and some forms of cerebellar ataxias.

Keywords: Adrenoleucodystrophy, Alzheimer's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, cerebrotendineous xanthomatosis, fragile-x-associated tremor ataxia syndrome, Friedreich ataxia, frontotemporal dementia, Huntington's disease, Kearns Sayre syndrome, Lewy body dementia, MELAS, non-pharmacological treatment, Parkinson's disease, pharmacological treatment, Pick disease, prion disease, progressive supranuclear palsy, Refsum's disease, spinocerebellar ataxias.

2.1. INTRODUCTION

Neurodegenerative diseases are caused by a progressive loss of neural cells, leading to dysfunction of specific areas of the nervous system [1]. Although

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epidemiology of neurodegeneration is not a simple task, Alzheimer's disease and Parkinson's disease are the most common neurodegenerative diseases [2, 3]. Epidemiological studies have shown that Lewy body dementia is the third most frequent [4, 5]. Much less common are frontotemporal dementia, Huntington's disease, amyotrophic lateral sclerosis, progressive supranuclear palsy, spinocerebellar ataxias, Pick disease and prion disease [3]. Neurodegenerative disorders are an important cause of mortality and morbidity in the elderly and have great impact in our society. Disorder's like Alzheimer's and Parkinson's disease generally lead to behavioral alterations like depression and psychosis. Although the understanding of the molecular mechanisms underlying neuronal loss has progressed in accelerated pass, there is no currently available therapy that is capable to avoid the progression of these disorders.

Current pharmacological therapies offer limited and transient symptomatic benefits with very little impact, if any, in modifying the course of these diseases. The known pathogenesis of neurodegenerative diseases is multifactorial, thus making the conventional monofactorial approach ineffective in slowing the progression of neuronal loss. Drugs acting in a single receptor tend to present resistance because alterations in number and affinity of receptors can hinder the efficacy. The association of environmental and behavioral modification with drugs directed to multiple targets would more likely be successful [6].

2.2. ALZHEIMER'S DISEASE

a) Pathophysiology of Alzheimer's Disease

The main proposed hypothesis to explain the pathophysiology of Alzheimer's disease is the amyloid cascade [7]. According to this hypothesis, abnormal production or clearance of amyloid β peptide (Aβ) results in extracellular amyloid deposition. Amyloid deposition causes hyperphosphorylation of the tau protein generating neurofibrillary tangles (NFT), excitotoxicity, inflammation, apoptosis and cell death. These events are related to neurotransmitter deficits, which are partly responsible for some clinical manifestations. The cleavage of the amyloid precursor protein (APP) may occur by two pathways: sequential cleavage by alpha and gamma-secretase leading to formation of soluble P3 peptide; or sequential cleavage by beta and gamma-secretase leading to the formation of insoluble Aβ peptide. P3

protein does not form insoluble aggregates, while Aβ deposits into amyloid plaques that are found in Alzheimer's brains. Supporting this hypothesis, familial autosomal dominant forms of Alzheimer's disease that are responsible for less than 5% of all cases occur as a result of gene mutations in APP and presenilin (gamma-secretase) genes (1 and 2) that increase Aβ formation [8].

Another hypothesis is that pathological aggregation of the microtubule-associated protein tau is the primary event [9]. NFTs are produced by hyperphosphorylation of protein tau that is responsible for stabilizing the axonal cytosqueletton, disrupting the axonal transport and leading to cell death. Mutations of the tau gene microtubule associated protein tau (MAPT) cause familial frontotemporal dementia with Parkinsonism, in which amyloid plaques are not found, suggesting that abnormal tau is sufficient for neurodegeneration [9]. Recent data show that risk factors to cardiovascular disease are also risk factors for Alzheimer's disease, suggesting a vascular contribution for the pathogenesis of the disease. In fact, coexistence of ischemic lesions, amyloid plaques and NFT increases the risk of dementia (for additional information see Chapter 7). Current pharmacological treatments for Alzheimer's disease are summarized in Table **1**.

b) Cholinesterase Inhibitors

Patients with Alzheimer's disease have deficit in acetylcholine production which is responsible for some of its symptoms. Cholinesterase inhibitors block the cholinesterase enzyme that degrades acetylcholine in the synaptic cleft, thus increasing acetylcholine levels. Cholinesterase enzyme has two forms: acetylcholinesterase and butyrylcholinesterase. Cholinesterase inhibitors improve Alzheimer's disease symptoms without avoiding the progression of its clinical course. In this regard they are considered symptomatic treatments. The best outcomes are on neuropsychiatric alterations, helping to alleviate some symptoms and preventing others. Cholinergic side effects are dose-dependent and include anorexia, nausea, vomiting, diarrhea, abdominal pain, dizziness, fatigue and muscle cramps. If side effects persist and response is not satisfactory, clinical practice suggests that switching to a different cholinesterase inhibitor may be an option. In case of poor clinical response, another possibility is to add memantine without discontinuing the initial medication. Discontinuation of treatment is an

option when patient or caregiver decides so, when there is poor adherence to treatment, no verifiable response, severe side effects and progression to a stage of the disease where there is no benefit of this treatment and presence of comorbidities that make its use inappropriate [7].

Donepezil is the most used cholinesterase inhibitor worldwide. Donepezil inhibits acetylcholinesterase increasing the availability of acetylcholine. According to a systematic review, patients treated with donepezil showed significant benefits on cognitive, global, functional and behavioral parameters in a dose dependent fashion [10]. Side effects were mild and transient including nausea, vomiting, diarrhea, dizziness, fatigue, nightmares and anorexia. Rivastigmine inhibits both acetylcholinesterase and butyrylcholinesterase. It is available in oral and transdermal presentations. Transdermal presentations aim minimizing side effects and facilitating administration in less collaborative patients. A systematic review showed that rivastigmine improves cognitive, global and functional but not behavioral outcome measures [11]. Benefits were observed in people that used 6 to 12 mg daily. Side effects included nausea, vomiting, diarrhea, anorexia, headache, syncope, abdominal pain and dizziness [11]. The highest transdermal rivastigmine dose (12mg daily) was as effective as oral rivastigmine but with less side effects [12, 13]. In some patients transdermal rivastigmine is associated with skin intolerance [13]. Galantamine reversibly inhibits acetylcholinesterase and binds to nicotinic receptors enhancing cholinesterase transmission [7]. A Cochrane review showed significant effects of galantamine on cognitive, global, functional and behavioral parameters [14]. A trial that included mixed vascular and Alzheimer's dementia showed a significant benefit of galantamine with 16 to 24mg daily [15]. Typical cholinergic side effects are present including nausea, vomiting, diarrhea, dizziness and anorexia [7].

An important pitfall for these treatments, however, is that mild-to-moderate AD patients receiving cholinesterase inhibitors show improvement on cognitive measures that reach its maximum at 6 months and decays to baseline at approximately 12 months. Functions that are lost are generally not recovered. The temporary effects of these treatments arrive because anticholinesterase drugs rely on intact cholinergic terminals, which continue to degenerate as the disease progresses. For this reason direct muscarinic and nicotinic agonists are being

developed. Some studies show that direct muscarinic M1 stimulation can also decrease Aβ levels possibly slowing the disease progression [16].

c) Memantine

Memantine is a noncompetitive NMDA glutamate receptor antagonist. Its use aims to avoid glutamate-related excitotoxicity. A systematic review showed significant treatment benefits on cognitive, global and functional aspects. One study showed that adding memantine to donepezil treatment leads to additional improvement of cognitive, functional and global parameters [17]. In moderate-to-severe Alzheimer's disease memantine contributes to stabilize cognitive and functional symptoms for about 6 months [7, 18].

d) Cardiovascular Risk Factors

According to clinical trials, treatment of hypertension was associated with better cognitive outcomes in patients with dementia although not associated to a reduced incidence [19]. The same association was not observed for cholesterol lowering medications. However, results from these clinical trials are not conclusive due to methodological limitations [7].

e) Comorbidities

Patients with Alzheimer's disease suffer from many comorbidities, such as depression, cerebrovascular disease and chronic pain. Adequate diagnosis and treatment of these conditions contribute to improvement of the clinical picture [7, 18].

f) Disease-Modifying Treatments

Based on prior animal data, interfering with APP cleavage is a possible target for modifying disease progression [7]. Multiple targets have been defined acting in α-secretase and γ-secretase. Semagacestat is a γ-secretase inhibitor which decreases Aβ40/42 plasma concentration. Phase III studies of semagacestat did not slow disease progression and worsened cognitive function [7].

Aβ peptides have different sizes and Aβ42, consisting of 42 amino acids, is considered a more toxic isoform. Aβ42 lowering agents, such as tarenflurbil, can

favor the production of shorter forms thus reducing the progression of the disease. Unfortunately tarenflurbil did not show beneficial effects in phase III studies [7]. Stimulating α-secretase can move APP cleavage towards formation of non-amyloidogenic peptides. There are ongoing studies with drugs that increase α-secretase and decrease β-secretase activity [7].

Another possible target to reduce Aβ burden is the removal of polymerized Aβ peptides. Tramiprosate (homotaurine) is a drug that prevents Aβ aggregation by binding to soluble Aβ and preventing its aggregation. It was not very effective in clinical trials but some studies showed slowing of hippocampal atrophy. It has since been approved as a drug to prevent memory loss, although some data suggest it may promote tau protein aggregation. Another mechanism proposed is the removal of ions that are necessary for Aβ aggregation, like copper and zinc. Substances that interfere with this mechanism are being tested. Other drugs to prevent aggregation that are being studied are epigallocatechin and scyllo-inositol.

Aβ peptides can also be removed by endogenous clearance. Clearance of Aβ from the brain occurs by multiple processes, like drainage along perivascular basement membrane into the cerebrospinal fluid, transport across vessel walls into circulation, P-glycoprotein efflux pump, sequestration of Aβ by soluble low-density lipoprotein in the circulation to promote efflux of soluble Aβ out of the central nervous system, microglial phagocytosis and enzyme mediated degradation [8]. In order to promote clearance of Aβ peptides, passive immunization with monoclonal antibodies or donor polyclonal immunoglobulin are also being tested [16]. Active immunization with AN172 (the first vaccine used in humans) had to be terminated due to an aggressive autoimmune response leading to meningitis and death [7]. Other vaccines that appear to be safer are now in study. Despite many attempts, no agent aiming to reduce amyloidogenesis has obtained positive results so far [7].

Agents targeted to other mechanisms are also being studied. The most important enzyme for the phosphorylation of tau protein is GSK3. Valproic acid and lithium can inhibit GSK3 to some degree, but initial studies are not encouraging. Other agents being tested are vitamin B3 and methylene blue. Mitochondrial stabilization with latrepirdine presented unsuccessful results. Inflammation, oxidative stress and excitotoxicity are other possible targets [7].

g) Cognitive Training

Cognitive training has been proposed as a possible mean of preventing cognitive decline as well as a treatment for Alzheimer's disease and other dementias. Cognitive training is a set of strategies directed to improve cognition. Although previous research has proved the benefit of cognitive training of healthy elderly for cognitive functions like memory, attention and reasoning, results for patients with cognitive impairment are more conflicting. Cognitive rehabilitation techniques for patients with mild-to-moderate Alzheimer's disease directed to implicit learning are being tested with significant benefits. Systematic reviews concluded that cognitive training is effective for Alzheimer's patients although with a moderate effect [20, 21]. Other studies reported less favorable results or no effect at all [22]. Brain training games have been proposed as an alternative strategy, which do not require a therapist and are less expensive. Some studies have shown the benefits of specific brain training software like Brain Test Britain, Memori65+ and Nintendo Brain Training that were effective for some individuals but generally ineffective for improving cognition in younger adults. Their efficacy needs further evaluation for older adults and mild dementia patients [22, 23].

Clinical experience shows that informal cognitive training is obtained when patients with Alzheimer's disease are not removed from society and family. Social contact, performing simple daily tasks and a stimulating environment can help patients to develop and maintain cognitive skills [22, 23].

h) Sleep Disturbances

Sleep and wake disturbances are common occurrences in Alzheimer's disease [24]. Patients with Alzheimer's disease have sleep disturbances similar to age matched elderly, but generally more severe and difficult to treat [22]. The most frequent are circadian rhythm disturbances, night wandering, sundowning and sleep apnea. Circadian rhythm disturbances are probably due to neuronal loss in the hypothalamus that occurs very early in the neuropathological evolution. Difficulty to synchronize circadian biological clock is probably related to confusion episodes, night wandering and sundowning [24]. Patients with Alzheimer's disease have typically reduced percentage and duration of REM sleep, which is probably related to loss of cholinergic neurons and memory deficit

[25]. Cholinesterase inhibitors can increase the amount of REM sleep which is related to cognitive and behavioral improvement [25]. Some studies suggest that the incidence of obstructive sleep apnea is higher in Alzheimer's patients and that there is a genetic link between both conditions [26]. It was described that Alzheimer's patients treated with CPAP (Continuous Positive Airway Pressure) show cognitive improvement [27]. A recent clinical trial showed that donepezil was beneficial for sleep apnea in Alzheimer's patients, improving apnea-hypopnea index and oxygen saturation but the mechanism explaining this improvement is still unknown [26].

Table 1: Pharmacological Treatment for Alzheimer's Disease

Substance	Mechanism	Use
Donepezil [7, 10, 18, 19, 28]	Inhibits acetylcholinesterase	Improve cognitive, functional, global and behavioral outcome measures
Rivastigmine [7, 11, 12, 13, 18, 19, 28]	Inhibits acetylcholinesterase and butyrylcholinesterase	Improve cognitive, functional and global outcome measures
Galantamine [7, 14, 15, 18, 19, 28]	Inhibits acetylcholinesterase Agonist of nicotine receptor	Improve cognitive, functional, global and behavioral outcome measures
Memantine [17, 29]	NMDA noncompetitive glutamate receptor antagonist	Improve cognitive, functional and global outcome measures
Homotaurine (Tramiprosate, Vivimind) [8, 9, 16, 30]	Prevents Aβ aggregation	Modest effect as memory loss-preventing nutraceutical. Reduces hippocampal atrophy [8, 9, 31].
Caprylidene (Axona) [8, 9, 32, 33]	Dietary supplement: metabolized into ketone bodies	Caprydilene is specifically indicated for the clinical dietary management of the metabolic processes associated with mild to moderate Alzheimer's disease.

2.3. PARKINSON'S DISEASE

a) Pathophysiology of Parkinson's Disease

Parkinson's is a chronic neurodegenerative characterized by resting tremor, bradykinesia, cogwheel rigidity and postural instability. Currently there is no curative or disease-modifying treatment. Disorders of movement in Parkinson's disease result from a deficiency of dopamine stimulation of basal ganglia. This is mainly due to the degeneration of dopamine-producing neurons of substantia nigra. Deficiencies of other neurotransmitters are present including serotonin and

norepinephrine. The two principal pathways within the basal ganglia are direct and indirect. In the direct pathway, the cerebral cortex inputs to the striatum, which in turn projects inhibitory GABA and substance P efferents into the globus pallidus and substantia nigra. The globus pallidus projects GABA efferents to the thalamus, which sends excitatory glutamatergic efferents to the cortex. In the indirect pathway, the striatum projects inhibitory GABA and enkephalin efferents to globus pallidus, which projects GABA efferents to the subthalamic nucleus. The subthalamic nucleus sends excitatory glutamatergic projections to the internal globus pallidus and the substantia nigra. Pallidus and substantia nigra neurons send inhibitory GABA projections to the thalamus thereby inhibiting thalamic stimulation of the cortex. Direct and indirect pathways have opposite effects on the thalamic input to the cortex. Dopamine is produced in pars compacta of substantia nigra. Dopamine acts both in the direct and indirect pathways, but dopamine loss results in an overall reduction of thalamic excitatory output to the cortex (for additional information see Chapter 8) [34].

Clinical aspects of Parkinson's disease are variable due to this complex brain circuitry. Evolution of Parkinson's disease can by differentiated in tremor-predominant and postural instability-predominant forms. Generally, postural instability-predominant form is more incapacitating and has worse prognosis [34]. Currently used pharmacological therapies for Parkinson’s disease are summarized in Table **2**.

b) Levodopa

Levodopa is the most effective pharmacological treatment for Parkinson's disease. Generally speaking, levodopa therapy represented a great progress in Parkinson's disease management. Quality of life of patients increased and their mortality rate is close to normal elderly. Most Parkinson's patients die from other complications like cardiovascular and infectious diseases. Falls with hip fractures are caused by postural instability and are related to increased mortality [34]. Initially, levodopa provides a stable response but in long-term its benefit declines and fluctuations in motor response occur. Long-term studies suggest that it is better to start therapy using selegiline and dopamine agonists and to add levodopa when the effects of the former are not adequate anymore [34].

Most levodopa is metabolized before it can cross the blood-brain barrier. By this reason, levodopa is generally associated with a decarboxylase inhibitor to prevent peripheral conversion to dopamine thus reducing side effects and increasing its bio-availability [34]. The most used associations between levodopa and decarboxylase inhibitor are levodopa/carbidopa and levodopa/benserazide. Controlled-release preparations have been developed in order to produce fewer fluctuations in plasma levels and improve therapeutic response [34]. Peripheral side effects of levodopa include nausea, vomiting, hypotension and cardiac dysrhythmias. Careful titration of the dose is necessary to reduce side effects. Central side effects are confusion, delirium and behavioral changes. Some unconfirmed studies suggest that metabolites of levodopa can harm dopaminergic neurons [35]. Complications of levodopa treatment include dyskinesias, fluctuations and psychiatric disturbances. Clinical fluctuations include on-off syndrome. In this case, the effective period of levodopa response becomes progressively shorter. In on-off syndrome, there is sudden loss of drug response (off) followed by sudden return (on). Some authors advocate that fluctuations and dyskinesia can be managed by increasing the frequency of administration. Generally, clinicians resort to dopamine agonists and controlled release formulations in order to reduce these complications. The use of levodopa in early stages of disease increases the likelihood of complications such as dyskinesia and fluctuations [34-36]. A study evaluated whether levodopa/carbidopa/entacapone (Stalevo) delays the development of dyskinesia compared to levodopa/carbidopa but contrary to what was expected, time to development of dyskinesia was shorter and incidence of dyskinesia was higher in the entacapone-treated group. Entacapone-treated had slightly better symptom control. For this reason entacapone is not currently recommended for early Parkinson's disease [34, 36, 37].

c) Dopamine Agonists

Dopamine agonists are used as adjunctive treatment for Parkinson's disease. Available dopamine agonists are ergot alkaloids that act in postsynaptic receptors. They help the management of rigidity and bradykinesia. Association between dopamine agonists and levodopa results in fewer fluctuations [34, 38]. Dopamine agonists provide moderate symptomatic efficacy and are effective as monotherapy before levodopa is required [34]. Double-blind controlled trials comparing initial pramipexole and ropinirole with initial levodopa found no evidence favoring one

of these strategies over the other although dyskinesias were slightly less prevalent in pramipexole and ropinirole-treated groups [34, 38].

d) Anticholinergics

Anticholinergic drugs improve symptoms of Parkinson's disease by blocking cholinergic stimulation. They improve tremor but have little or no effect on rigidity and bradykinesia. The most used anticholinergic drugs are biperiden and trihexylphenidil. Side effects include memory loss, hallucinations, xerostomia and urine retention [34, 38].

e) Amantadine

Amantadine is an antiviral drug that serves as an NMDA antagonist, improving parkinsonian symptoms by dopaminergic and anticholinergic actions. It can be helpful as an adjunctive treatment in order to reduce fluctuations and dyskinesia [34, 38].

f) Selegiline

Selegiline is an inhibitor of monoamine oxidase, type B (MAO-B) thought to slow the progression of Parkinson's disease. MAO-B inhibition reduces degradation of dopamine increasing its availability. It also protects neurons by inhibiting the production of free radicals. Selegiline can be used in the early stage of disease in order to delay the introduction of dopaminergic drugs [34, 36, 37]. A clinical trial showed that after 5 years of combined therapy with levodopa, the placebo group was 35% worse than the selegiline-treated and its levodopa doses were 19% higher. Generally, most studies suggest that earlier initiation and longer duration of selegiline treatment improves long-term outcome [34, 38].

g) Advanced Parkinson's Disease

The pharmacological approach in advanced Parkinson's disease remains a difficult issue. The most common motor complications in advanced Parkinson's disease are motor fluctuations and dyskinesia [34, 38]. Motor fluctuations occur in the form of on-off phenomenon, unpredictable off and failure of on. The two main types of dyskinesia are peak dose dyskinesia and diphasic dyskinesia. Progression of disease associated to short half-life of levodopa leads to motor fluctuations after 4

to 6 years of treatment in 40% of patients [38]. Over time, the duration of benefit after a single dose of levodopa shortens. The pathophysiologic explanation is that progressive neuronal loss leads to reduced nerve terminals in the striatum reducing dopamine storage capacity. Fluctuations in plasma levodopa levels are no longer compensated by the striatum resulting in irregular stimulation of dopamine receptors that causes long-term changes in neurotransmitter pathways. Many strategies have been proposed to treat motor fluctuations. The most simple is shortening the interval between levodopa intakes but it can worsen dyskinesias and long-term drug outcomes [38]. Another strategy is the co-administration of catechol-O-methyltransferase (COMT) inhibitors. Currently available COMT inhibitors are entacapone and tolcapone. These drugs increase bioavailability by decreasing the peripheral cleavage of levodopa and increasing its half-life. Tolcapone is hepatotoxic [38]. Entacapone reduces on-off fluctuations and can be useful in advanced Parkinson's disease, improving the patient's quality of life. Continuous delivery of dopamine agonists is available in 24-hour formulation of ropinirole and transdermal rotigotine that can help to control wearing-off phenomenon. Atypical antipsychotics can help the treatment of dyskinesia. Clinical trials showed the efficacy and safety of clozapine for this indication. Olanzapine, risperidone, quetiapine and aripiprazole have also been tested with positive results [38].

h) Surgery

Surgical treatments for Parkinson's disease were developed before the introduction of levodopa. Later they were used to overcome some difficulties in medical management of advanced Parkinson's disease. Stereotactic ablations focused on the pallidothalamic pathway including the globus pallidus and the thalamus. Selective lesions of the internal globus pallidus improved dyskinesias and other motor symptoms but there was always the risk of permanent motor deficits. Subthalamic lesions were also effective but caused hemibalism in some patients [39].

Deep brain stimulation was first used to check a specific area before surgical lesion. Later it was developed as a reversible alternative to stereotactic ablation. Bilateral deep brain stimulation of the subthalamic nuclei and globi pallidi interni has been studied in randomized controlled trials. Significant improvement of

motor symptoms was described in patients with deep brain stimulation of the subthalamic nuclei and globi pallidi interni, including motor fluctuations and tremor. Unilateral stimulation can be considered in specific cases. The amount of improvement depends on individual conditions. Stimulation of pedunculopontine nucleus has also been selected as a target but outcomes are variable. Some non-motor symptoms, like cognitive deterioration and mood disorders, may also improve, partly because reduction of drug treatment. Mechanisms by which deep brain stimulation is effective are not fully understood [39].

i) Physical and Phonoaudiologic Therapy

The therapeutic spectrum for Parkinson's disease include multiple modalities besides pharmacological and surgical treatment: psychological, phonoaudiologic and physical therapy [34, 40]. Physical therapy is directed to avoid restriction of motion range and loss of aerobic capacity. It improves strength and flexibility. Low volume and pronunciation problems can be improved by speech therapy. When improvement is not possible, adaptive equipment is needed like walkers and bed rails. Some studies show modest effects of physical exercise [34, 40]. There is evidence that regular exercise can prevent Parkinson's disease and other neurodegenerative diseases as well as slow its progression [40].

j) Parkinson's Disease with Dementia (PDD)

Cognitive impairment is recognized as part of Parkinson's disease. Most patients develop some degree of cognitive impairment 15 years after diagnosis. Dopamine pathway is not the only one affected in Parkinson's disease. Serotonergic deficit contributes to mood disorder, noradrenergic to impaired attention and mood, cholinergic to deficit in memory, attention and executive functions. In Parkinson's disease there is an important loss of cholinergic neurons in the nucleus basalis of Meynert. Cholinergic loss was more extensive in dementia with Lewy bodies and Parkinson's diseases than in Alzheimer's disease [41].

Clinical trials suggested that anticholinesterase drugs including donepezil, rivastigmine and galantamine provide improvement in cognition and behavioral symptoms without impairment of motor functions in PDD. A placebo controlled trial showed that patients on rivastigmine presented sustained improvement for 12 months in cognitive scales and attention with some adverse effects: nausea and vomiting. Worsening of parkinsonian symptoms was more frequent in rivastigmine

group (27.3%) than in the placebo group (15.6%) [41]. Rivastigmine was approved for PDD in USA and Europe. A more recent trial showed that donepezil is effective against cognitive deficit in PDD improving mini-mental state examination (MMSE) scores, attention and verbal fluency. Memantine has not yet been tested in a large controlled trial but preliminary results show good tolerability and some benefits [41].

Table 2: Pharmacological Treatment for Parkinson's Disease

Substance	**Mechanism**	**Use**
Levodopa/Carbidopa Levodopa/Benserazide [34-38, 42-45]	Dopamine replacement	First course of treatment, manage major parkinsonian symptoms
Levodopa/Carbidopa/ Entacapone [34-38, 42-45]	Dopamine replacement + COMT inhibitor	Secondary course, prolong effectiveness of levodopa
Pramipexole (or extended-release) [34-38, 42-45]	Dopamine agonist	Alone or with levodopa; manage major parkinsonian symptoms
Pergolide [34-38, 42-45]	Dopamine agonist	Alone or with levodopa; manage major parkinsonian symptoms
Bromocriptine [34-38, 42-45]	Dopamine agonist	Alone or with levodopa; manage major parkinsonian symptoms
Apomorphine (injection) [34-38, 42-45]	Dopamine agonist	With levodopa therapy to treat "off" periods
Rotigotine (transdermal) [34-38, 42-46]	Dopamine agonist	Alone or with levodopa; manage major parkinsonian symptoms
Ropinirole [34-38, 42-46]	Dopamine agonist	Alone or with levodopa; manage major parkinsonian symptoms
Benztropine mesylate [42-46]	Inhibits acetylcholinesterase and butyrylcholinesterase	Secondary medication for tremor
Trihexyphenidyl [44, 46]	Anticholinergic	Secondary medication for tremor
Biperiden [44, 46]	Anticholinergic	Secondary medication for tremor
Selegiline [34-38, 42-45]	MAO-B inhibitor	Alone or with levodopa; controls brain metabolism of dopamine.
Rasagiline [42-45]	MAO-B inhibitor	Alone or with levodopa; controls brain metabolism of dopamine.
Amantadine [42-45]	NMDAglutamate antagonist	Secondary medication for tremor, rigidity and dyskinesia
Entacapone [42-45]	COMT inhibitor	Secondary medication; delays wearing off by prolonging effectiveness of levodopa
Tolcapone [42-45]	COMT inhibitor	Tertiary medication for motor fluctuations; limited in use to those who have exhausted other treatment options
Rivastigmine [41]	Anticholinesterase	Parkinson's disease with dementia

2.4. DEMENTIA WITH LEWY BODIES

Dementia with Lewy bodies is characterized by progressive cognitive impairment. There is a debate whether dementia with Lewy bodies and Parkinson's disease with dementia are separate entities. A practical 12-month rule is frequently used: onset of dementia within 12 months of parkinsonism is indicative of dementia with Lewy bodies, after more than 12 months of parkinsonism is suggestive of Parkinson's disease with dementia [47]. At onset, attention deficit and executive dysfunction predominate. Later, memory is affected although in a milder way than in Alzheimer's disease. Daytime sleepiness and visual hallucinations are early symptoms. Parkinsonian symptoms appear throughout the evolution of the disease, predominating rigidity, bradykinesia and gait disturbances. REM sleep behavior disturbance is characteristic and may precede cognitive disturbance. Hypersensitivity to neuropleptics is observed characteristically as an exacerbation of parkinsonian symptoms. Dysautonomia is manifest in orthostatic hypotension, syncope and urinary incontinence. Psychiatric disturbances are frequent, including depression, agitation, hallucinations and aggressiveness (for additional information see Chapter 12) [48]. Currenlty used pharmacological treatments for other neurodegenerative diseases are summarized in Table **3**.

Treatment is complex, involving cognitive and motor symptoms. In Lewy body dementia, loss of cholinergic neurons is more accentuated than in Alzheimer's disease. The anticholinesterase drugs donepezil, rivastigmine and galantamine have shown positive results for cognitive and psychiatric symptoms including apathy and hallucinations. A comparative study has shown similar efficacy for these three drugs with no significant deterioration of parkinsonian symptoms. Some studies have also shown a beneficial effect of memantine for cognitive and psychiatric symptoms [49].

Levodopa is well tolerated by patients with Levy body dementia. Low doses generally do not affect sleep quality and behavioral aspects. High doses can provoke hallucinations and psychotic manifestations. Levodopa treatment is not as effective as in Parkinson's disease and long term studies show a lesser motor benefit. Dopamine agonists are less useful in dementia with Lewy bodies than in Parkinson's disease due to side effects like mental confusion, hallucinations and psychotic symptoms [48].

Orthostatic hypotension can be exacerbated by levodopa and dopaminergic agonists. Pharmacological adjustment and increased liquid intake are useful options. Behavioral symptoms are frequent and more difficult to treat than in Alzheimer's patients. Antipsychotic hypersensitivity is present in Lewy body dementia, generally causing sedation, immobility, rigidity and postural instability. Considering this fact, dosage of antipsychotic drugs must be smaller and slowly titrated. Quetiapine and clozapine are better tolerated than risperidone and olanzapine. Low doses of quetiapine is the most adequate and efficacious antipsychotic treatment for these patients [48].

2.5. FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia is not a single entity. It is a group of disorders in which there is a tendency towards asymmetric cortical atrophy in anterior structures of the frontal and temporal lobes, sparing the parietal lobe structures. It includes three clinical pictures. Behavioral frontotemporal dementia is characterized by apathy, disinhibition and loss of empathy leading to socially inappropriate behavior. Patients do not take care of diet, personal hygiene, become impulsive and careless. Neuroimaging shows focal atrophy of the nondominant orbitofrontal, medial frontal and anterior insular cortex. The typical histopathological feature is the presence of frontotemporal lobar degeneration associated tau aggregates (FTLD-tau), FTLD-TAR DNA-binding protein (FTLD-TDP) and FTLD fused-in-sarcoma protein (FTLD-FUS) (for additional information see Chapter 12). Semantic variant of frontotemporal dementia is characterized by word-finding difficulty due to a loss of semantic meaning. Additional symptoms are impaired face recognition and inflexible behavior. Neuroimaging reveals temporal pole atrophy worse in dominant hemisphere. FTLD-TDP is the usual pathophysiological finding. Progressive nonfluent aphasia variant is characterized by slow and difficult speech with dysarthria and phonemic errors. Basic comprehension is preserved but there is difficulty understanding complex sentences. Motor features are orobucal apraxia, tremor and rigidity. Neuroimmaging reveals asymmetric dominant frontal opercular and anterior insular atrophy with involvement of ipsilateral striatum and subcortical nuclei. Neuropathology reveals FTLD-tau in these patients [50].

There is no FDA-approved therapy for frontotemporal dementia. For this reason off-label medication prescription is common. Serotonin reuptake inhibitor antidepressants and antipsychotic medications are often used [50]. In an open-label study patients treated with fluoxetine, sertraline or paroxetine showed a reduction in depressive symptoms, disinhibition and compulsions [50]. Trazodone was effective in a placebo-controlled trial to control behavioral symptoms [50]. Antipsychotic medications risperidone and olanzapine have been tested with some benefit [50]. Patients with frontotemporal dementia are very sensitive to extrapyramidal symptoms of antipsychotic medications. In frontotemporal dementia there is a relative preservation of cholinergic neurons suggesting that anticholinesterase drugs are not effective to improve cognitive function. On the other hand, the improvement of psychiatric symptoms by anticholinesterase drugs in Alzheimer's disease raised the possibility of similar effects in frontotemporal dementia. The lack of evidence for a benefit of galantamine and rivastigmine and the possibility that donepezil may worsen the behavioral variant of frontotemporal dementia led to the recommendation to avoid cholinesterase inhibitors in frontotemporal dementia. On the other hand, clinical trials showed that memantine was beneficial for behavioral symptoms in frontotemporal dementia even though more studies are needed to verify the benefit of memantine as a symptomatic therapy or a disease modifying therapy [50].

2.6. PICK'S DISEASE

Pick's disease accounts for 0.4% to 2% of dementia cases and its onset is before 65 years age, with no gender preference. It progresses with aphasia and behavioral disturbances, rapidly causing disability and death. Neuroimaging shows atrophy of the frontal and anterior temporal lobes, confirmed by PET and MRI that reveal a marked reduction in perfusion of these areas. The neuropathologic characteristic of this disease are Pick bodies and Pick cells. Pick bodies are round, well-circumscribed cytoplasmic eosinophilic and argyrophilic inclusions. Pick cells are large swollen ballooned neurons with vacuolated cytoplasm. There is some terminological confusion because some authors use the term Pick's disease only if Pick bodies are present whereas others consider the clinical picture and focal atrophies as hallmarks. Pick's disease is caused by accumulation of mutations in the protein tau, altering its interaction with microtubules and leading to its

abnormal phosphorylation and polymerization. In this regard, Pick's disease can be classified as a tauopathy [51, 52]. There is no specific pharmacological treatment for Pick's disease. Antidepressants and antipsychotics may help to control behavioral disturbances that can be dangerous to patient and others. Some patients with Pick's disease receive the same medications used to treat Alzheimer's disease such as anticholinesterase inhibitors and memantine, but there is no conclusive evidence that these medications can help. Speech and occupational therapy may improve communication and movement [51, 52].

2.7. PROGRESSIVE SUPRANUCLEAR PALSY

Progressive supranuclear palsy is a neurodegenerative tauopathy that manifests in various syndromes. The classical phenotype, named Steele-Richardson-Olszewski syndrome, is characterized by bradykinesia, rigidity and tremor, modestly responsive to levodopa, which evolves with supranuclear gaze palsy, postural instability, reduced eyeblink and swallowing disorders. Cortical signs such as amnesia and aphasia tend to be mild in more typical forms. In some atypical forms, progressive apraxia of speech, nonfluent aphasia and impaired executive functioning may evolve. Neuroimaging may show the hummingbird sign, in which mid-saggital images look like a hummingbird. Frontosubcortical hypometabolism can be found by PET. There is currently no effective treatment for progressive supranuclear palsy. Symptomatic treatment includes levodopa, dopamine agonists and anticholinergic drugs which can help to improve rigidity and bradykinesia but are not as effective as in Parkinson's disease. Antidepressants may help to improve behavioral symptoms. Walking aids and exercises may be helpful. Gastrostomy may be necessary to treat swallowing disturbance. The main causes of death are pneumonia, fractures and head injuries caused by falls. Well treated patients can reach old age [53].

2.8. AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis is characterized by progressive muscle wasting in more than one segment of the neuroaxis producing progressive muscle weakness, fibrillations, stiffness, and pathological reflexes. Cognitive alterations were found in selected populations, typically presenting as subtle deficits in mental flexibility,

verbal fluency and abstract reasoning. The main neuropathological characteristic of amyotrophic lateral sclerosis is the formation of intraneuronal aggregates of phosphorylated neurofilaments. Neurofilaments are a component of neuron's cytoskeleton providing support to axonal radial growth. These findings suggest that disturbances in the metabolism of neurofilaments lie in the basis of the pathogenesis of amyotrophic lateral sclerosis. Degeneration of selective populations of motor neurons is observed, including supraspinal motor pathways, spinal motor neurons and brainstem. The classical sporadic form accounts for most cases. Familial amyotrophic lateral sclerosis accounts for less than 10% of cases and can be autosomal dominant or X-linked. More recently a relationship between amyotrophic lateral sclerosis and frontotemporal dementia has been demonstrated the by the discovery that TDP-43 and FTLD-FUS proteins are molecular markers in most amyotrophic lateral sclerosis [54, 55] (for additional information see Chapter 10).

Presently, there is no curative treatment for amyotrophic lateral sclerosis. Riluzole, the only FDA-approved drug for this disease, is an antiglutamatergic agent with some efficacy to prolong survival and slow disease progression. Some evidence shows that patients receiving riluzole present less neuronal loss. The most common adverse effects associated to riluzole are asthenia, nausea, dizziness, and a reversible elevation of liver enzymes [56]. Many other drugs are being tested: dexpramipexol, l-threonine, gabapentin, lamotrigine, nimodipine, TRH *etc*. Many related symptoms need to be addressed: malnutrition, respiratory disturbances, muscle cramps, sialorrhea, pain, fatigue, constipation, dysarthria, pseudobulbar affect, depression, anxiety and sleep disturbances. Physical and respiratory therapy may be helpful. Respiratory support is needed in advanced stages [53, 54].

2.9. HUNTINGTON'S DISEASE

Huntington's disease is a neurodegenerative genetic disorder with autosomal dominant inheritance. Its symptoms become evident in middle aged adults and include chorea, dystonia, Parkinsonism, tics, myoclonus, depression, apathy, anxiety, irritability, psychosis and cognitive deterioration. Genetic mutation results in the production of a mutant isoform of a protein called Huntingtin (Htt). Huntingtin is related to cell signaling but its mutant form becomes toxic to many

types of cells in the brain (for additional information see Chapter 9). There is no cure for Huntington´s disease. Tetrabenazine is the only FDA-approved drug for chorea and tics in Huntington's disease. Clonazepam may be useful in treating dystonia and myoclonus. Psychiatric symptoms can be treated with specific medications. Antidepressants like mirtazapine are recommended for depression. Atypical neuroleptics are recommended for psychosis and behavioral disturbances. As the disease progresses, the patient becomes less autonomous and multidisciplinary caregiving becomes vital. There are some evidence supporting the utility of physical, occupational and phonoaudiologic therapy [57].

2.10. CEREBELLAR ATAXIAS

Cerebellar ataxias are a group of hereditary neurodegenerative disorders characterized by progressive degeneration of the cerebellum, resulting in ataxia and incoordination of gait, hands, speech and eye-movements. Involvement of the spinal tracts is common, accompanied by diminished vibratory sense and hyperreflexia. Extrapyramidal signs, spasticity, cognitive impairment, polyneuropathy, ophtalmoplegia and epilepsy may occur. Autosomal dominant cerebellar ataxias are classified in 26 different subtypes. Not all types of the disease have similar prognosis and severity but it is always progressive and most patients will need a wheelchair in advanced stages. Friedreich ataxia (FA) and ataxia telangiectasia (AT) are the most common autosomal recessive cerebellar ataxias. Friedreich ataxia is characterized by ataxia, dysarthria, absence of deep tendon reflexes, corticospinal signs and has an early onset, before 25 years of age. Cardiomiopathy, distal scoliosis, distal muscle atrophy and diabetes are common features. The gene for FA (FRDA1) encodes frataxin, a mitocondrial protein. FA is thought to be caused by a mitochondrial dysfunction. Ataxia teleangectasia is characterized by oculomotor apraxia, ataxia, oculocutaneous telangiectases, choreoathetosis and dystonia. Immunodeficiency, hypersensitivity to ionizing radiation and predisposition to tumors are also present. Other autosomal recessive cerebellar ataxias are abetalipoproteinemia, Charlevoix-Saguenay ataxic syndrome, Marinesco-Sjögren syndrome, Cayman ataxia among others. Some autosomal recessive ataxias are part of a metabolic disorder including metachromatic leucodystrophy, cerebrotendineous xanthomatosis (CTX), sphingomielin storage disorder, GM1 gangliosidosis, Tay-Sachs disease, Wilson's disease, aceruloplasminemia, Refsum's disease, sialidosis, chorea-achanthocytosis

and leucoencephalopathy with vanishing white matter. Peripheral neuropathy is seen in AT and abetalipoproteinemia. Kaiser-Fleisher ring is characteristic of Wilson disease. Retinitis pigmentosa, anosmia polyneuropathy, cerebellar ataxia, deafness and ichthyosis are characteristic of Refsum's disease. Cerebrotendineous xanthomatosis is characterized by white matter lesions, juvenile cataracts, tendon xanthomas, chronic diarrhoea, ataxia, pyramidal signs, dementia, epilepsy and polyneuropathy. The most common x-linked cerebellar ataxias are adrenoleucodystrophy and fragile-x-associated tremor ataxia syndrome (FXATAS). Adrenoleucodystrophy is characterized by cerebellar ataxia and impaired adrenocortcal function, sometimes with cognitive decline. FXATAS is characterized by intension tremor, gait ataxia, Parkinsonism, polyneuropathy, dementia and dysautonomia. Some cerebellar ataxias are of maternal mitochondrial inheritance like Kearns Sayre syndrome and MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [58, 59].

In most cases medication is directed to control symptoms of tremor, stiffness, depression and spasticity. In autosomal dominant forms acetazolamide and also gabapentin have shown some effectiveness for cerebellar signs. In autosomal recessive subtypes, daily supplementation of vitamin E prevents neurodegeneration. In Refsum's disease, restriction of intake of phytanic acid may prevent onset of symptoms. Chenodeoxycholic acid stabilizes CTX. Baclofen and botulinum toxin may be used to treat spasticity. Dopaminergic and anticholinergic therapy may improve dystonia, tremor and bradykinesia. Muscle cramps can be relieved by benzodiazepines. Physical therapy can help patients to maintain autonomy and improve their quality of life [58, 59].

2.11. PRION DISEASES

Prion diseases are neurodegenerative diseases caused by an anomalous conformer of the prion protein [60-62]. The function of normal prion protein (PrP^{c}) is still uncertain but it has been related to a wide range of functions, based on knock-out mice studies, including cell signaling, protection against neuronal death, neuromuscular conduction, memory formation, regulation of the sleep-wakefulness cycle and immunomodulation [60-62]. Prion diseases are transmissible by inoculation of a misfolded prion-protein (PrP^{sc}) that induces

endogenous PrP^{c} to convert into pathologic infectious prion form, in a continuous process of exponential growth. PrP^{sc} produces aggregates that accumulate in infected tissue causing death. Alternatively, mutations in the endogenous PrP^{c} can cause familial forms of the disease [61]. Prion diseases can affect humans and animals. Human prion disease variants are Creutzfeld-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia and kuru [60, 61]. Prion diseases can be transmitted from animals to humans by ingestion of infected meat. There is no cure for prion diseases, partly because at the time it is detected most of the brain damage is already done. Presently there is no early diagnostic test for prion diseases. Future treatment targets are directed to avoiding conversion from normal to pathological PrP isoforms and to promote neuroprotection [61, 62]. The available symptomatic treatments include antidepressants, anxiolytics and neuroleptics that can promote temporary relief [60, 61].

Table 3: Pharmacological Treatment for Other Neurodegenerative Diseases

Condition	Drugs	Use
Dementia with Lewy Bodies [47-49]	No FDA approved for this form of dementia.	Off-label medications: Donepezil, rivastigmine and galantamine are effective for cognitive and psychiatric symptoms. Levodopa treatment is not as effective as in Parkinson's disease. Dopamine agonists cause psychiatric symptoms. Antipsychotic drugs cause sedation, rigidity and postural instability. Quetiapine is the most adequate antipsychotic.
Frontotemporal Dementia [50]	No FDA approved for this form of dementia.	Off-label medications: Fluoxetine, paroxetine, sertraline improve depressive symptoms. Trazodone improves behavioral symptoms. Risperidone and olanzapine may improve hallucination and psychosis. Anticholinesterase drugs are not indicated. Memantine can improve cognitive and behavioral symptoms.
Pick's Disease [51, 52]	No FDA approved for this form of dementia.	Off-label medications: Antidepressants and antipsychotics may be helpful to control behavioral symptoms. Anticholinesterase inhibitors and memantine can improve cognitive and behavioral symptoms.
Progressive Supranuclear Palsy (Steele-Richardson-Olszewski Syndrome) [53]	No FDA approved for this condition.	Off-label medications: Levodopa, dopamine agonists and anticholinergic drugs can improve rigidity and bradykinesia. Antidepressants may improve behavioral symptoms.
Amyotrophic Lateral Sclerosis [54-56]	Riluzole (antiglutamatergic)	Riluzole slow disease progression and prolong survival.

Table 3: contd...

Huntington Disease [57]	Tetrabenazine (vesicular monoamine transporter inhibitor)	Tetrabenazine improves chorea and tics. Off-label medications: Clonazepam may improve dystonia and myoclonus. Mirtazapine can improve depressive symptoms. Atypical neuroleptics may be useful for behavioral symptoms
Cerebellar Ataxias [58, 59]	Autosomal recessive types: vitamin E Refsum's: restriction of phytanic acid Cerebrotendineous xanthomatosis: chenodeoxycholic acid	Off-label symptomatic medications: Acetazolamide and gabapentin may improve cerebellar signs in autosomal dominant subtypes. Baclofen and botulinum toxin may improve spasticity. Dopaminergic and anticholinergic therapy may improve dystonia, tremor and bradykinesia. Muscle cramps can be relieved by benzodiazepines.
Prion diseases [60-62]	No FDA approved for these conditions.	Off-label medications: Symptomatic treatment include antidepressants, anxiolytics and neuroleptics

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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CHAPTER 3

"The Greatness of the Smallest Ones": The Most Valuable Attributes of Flies and Worms for the Study of Neurodegeneration

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Abstract: Over the last decades, a large number of experimental models have been developed to explore the mechanisms underlying neurodegenerative disorders. Invertebrate models of neurodegeneration, such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabtidis elegans*, have emerged as successful complementary systems to mammalian models, facilitating identification of relevant pathways and novel disease-associated genes. These organisms provide reliable systems for identifying genetic modifiers of neuropathologies and the interesting possibility of screening and testing potential drugs for treatments to prevent and/or alleviate disease symptoms.

This chapter will focus on the main experimental strategies used in *Drosophila melanogaster* and *Caenorhabtidis elegans* to study neurodegeneration. Insights from forward genetic approaches, transgenic models of human neurodegenerative disorders and studies of fly/worm homologs of human disease genes will be presented. The value of using invertebrate models for the study of neurodegeneration will be discussed, highlighting advantages and limitations associated with these studies.

Keywords: Alzheimer's disease, *Caenorhabtidis elegans, Drosophila melanogaster*, drug screening, forward genetics, fruit flies, genetic enhancers, genetic screen, genetic suppressors, Huntington's disease, invertebrate models, neurodegenerative diseases, neurodegeneration, neuronal death, Parkinson's disease, Polyglutamine diseases, reverse genetics, transgenic models, worms.

3.1. INTRODUCTION

Human pedigree analyses and gene linkage studies on families and populations with patterns of inherited neurodegenerative diseases have revealed genetic mutations responsible for disorders such as Alzheimer's disease, Amyotrophic

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lateral sclerosis, Huntington's disease and Parkinson's disease [1]. The identification of such mutations has opened a new field in neurosciences focused on deciphering the function of human disease genes in transgenic animals, with the ultimate aim of discovering the mechanisms of the associated neuropathology.

A large number of experimental disease models based on mutations in homologs of human disease-associated genes or direct expression of human neurodegenerative-associated disease genes have been established in the past years [1-8]. Traditional animal models, such as rodents, seem ideal disease model systems given their close evolutionary relationship with humans [6]. Yet, rodents are expensive and laborious to maintain. In this respect, invertebrate model organisms such as the fruit fly *D. melanogaster* and roundworm *C. elegans* are excellent alternatives for studying mechanisms of neurodegeneration. These simple model systems offer enormous experimental advantages, including short generation times, large number of offspring and low cost of maintaining in the laboratory. In addition, there exist powerful techniques for manipulating gene expression and function that have emerged in worms and flies, including the ability to perform large-scale genetic screens and genome-wide analyses of genetic interactions based on the modification of a given phenotype [9-11]. Finally, compared to vertebrates, *Drosophila* and *C. elegans* have the key benefit of relatively low genetic redundancy, as genes are usually present in one copy. This greatly simplifies genetics studies.

Despite these clear experimental advantages, it could be argued that invertebrate physiology is far too different from human physiology to directly translate findings in flies or worms to humans. Indeed, there are important differences that should be taken into consideration, *e.g.*, invertebrates have a less complex nervous system - with fewer neurons, glia and synapses than the human brain. Additionally, worms and flies lack some important components for many vertebrate pathological phenomena, such as inflammatory processes and neuronal myelination. Nevertheless, flies and worms share many fundamental cellular and molecular pathways with mammals, including those governing gene expression, cell cycle regulation, membrane trafficking, cellular toxicity and cell death. Importantly, the basic structural and functional components of the nervous systems are highly conserved between invertebrates and humans, including

Synaptic Proteins, ion channels and neurotransmitters (*e.g.* dopamine, acetylcholine, glutamate and GABA) [12-15]. This suggests that the fundamental mechanisms underlying neuronal viability and synapse function are evolutionarily conserved across species. Interestingly, various human pathological processes including cancer, ageing, neurodegeneration and infectious diseases also affect flies and worms. In this regard, the annotation of the *C. elegans* and *Drosophila* genomes revealed that more than 70% of the genes associated with human genetic disorders are present in these organisms (http://superfly.ucsd.edu/homophila/; [16-19]). Importantly, expression of several human neurodegenerative disease-associated genes in flies or worms recreates key neuropathological features of the disease including, in some cases, age-dependent neuronal degeneration, vulnerability of specific neuronal types and accumulation of proteins in abnormal aggregates [3, 20-26]. This demonstrates important parallels between these organisms and humans. These simpler model systems have served as platforms for identifying genes and pharmacological compounds that modulate the pathology, thus providing insights into the genetic and molecular basis of neurodegeneration [3, 4, 20-23, 25, 26]. In addition, unbiased genetic screens in invertebrates have uncovered genes not previously suspected to be involved in neuronal maintenance and viability, and interestingly, disrupting the function of some of these genes in more complex organisms also results in neurodegeneration. These findings validate such approaches as a meaningful way to identify conserved genes required to maintain nervous system integrity [24, 27, 28].

It is thus clear that *Drosophila* and *C. elegans* represent valuable model systems to study basic mechanisms governing neuronal dysfunction and death associated with human diseases.

3.2. *DROSOPHILA MELANOGASTER:* BASICS OF A POWERFUL GENETIC MODEL SYSTEM

Over a century of intensive research using *Drosophila melanogaster* to study complex biological processes has generated a vast knowledge about its genetics, anatomy and development. This small insect (of about 3 mm in length) lives near unripe and rotten fruit in nature (Fig. **1A**). Fruit flies develop through embryo, larval, and pupal stages followed by metamorphosis into the adult fly. They have

a short life cycle, of 10 days at 25 °C, and adults live for ~2–3 months, with the potential of producing hundreds of offspring. This is in contrast to rodent models, where only a few offspring are produced every 3 to 4 months. Although the fly nervous system (Fig. **1B**) has ~1 million-fold fewer neurons than the human brain, it is still capable of producing many complex behaviors, including mating behavior, intra-specific aggression, and learning and memory [29]. The adult fly brain contains around 200,000 neurons, including neurons involved in sensory perception, integration and motor output.

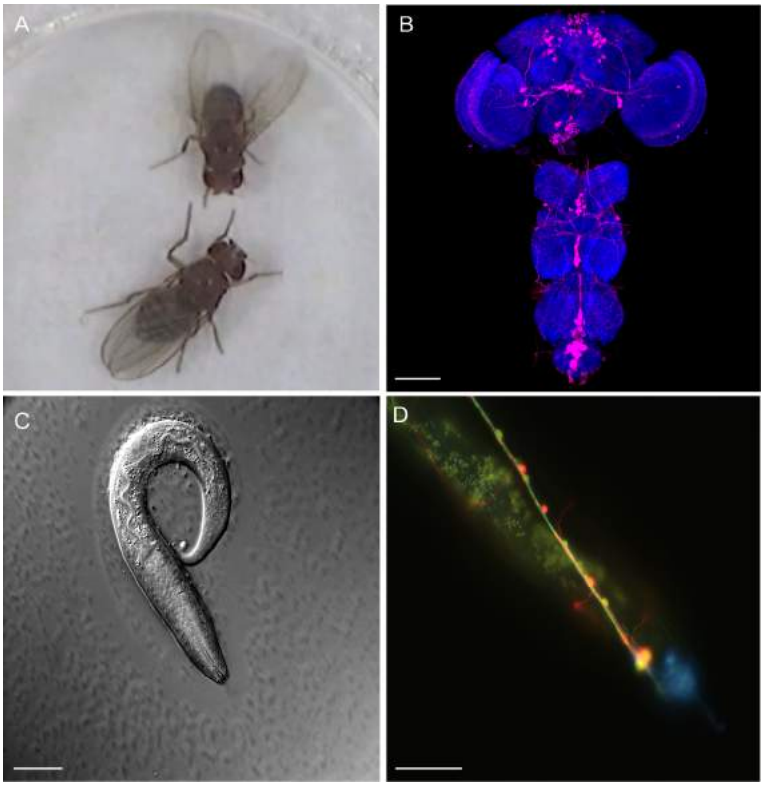

Figure 1: Fruit flies and worms: their anatomy and nervous system. (A) Image of an adult male and female *Drosophila melanogaster* performing the courtship ritual (top and bottom, respectively). (B) The *Drosophila* nervous system. The adult brain (top) and ventral nerve cord (bottom) are labeled with anti-nc82, a synaptic marker (shown in magenta). A sex-specific neuronal circuitry is visualised with anti-GFP antibody (shown in green). Scale bar: 100 µm. (C) *Caenorhabtidis elegans* (L1) larvae, soon after hatching. Scale bar: ~50 µm. (D) The *C. elegans* nervous system. Three types of motor neurons are shown in the ventral nerve cord of a triple transgenic animal: B-type motor neuron (DB; shown in green), A-type motor neuron (DA; shown in yellow) and D-type motor neuron (DD; shown in red). Scale bar: 50 µm. Images in A and B were taken by Carolina Rezával, University of Oxford. Pictures in C and D were kindly donated by M Gravato-Nobre, University of Oxford.

Sophisticated genetic techniques, such as random transposon tagging, site-specific transgenesis and recombination-mediated genetic engineering allow efficient manipulation of gene expression and function. There exist online databases containing valuable information regarding different aspects of *Drosophila* biology, including genes, mutations, phenotypes and available stocks (Flybase: http://flybase.org/), as well as nervous system anatomy (Flybrain: http://flybrain.neurobio.arizona.edu/; flymind) and development (the Interactive Fly: http://www.sdbonline.org/fly/aimain/1adult.htm).

3.3. *C. ELEGANS*: BASICS OF A CELLULARLY DEFINED MODEL SYSTEM

C. elegans is a free-living nematode of only ~1 mm in length that lives in temperate soil environments (Fig. **1C**). These roundworms have a rapid generation cycle (~3 days), short lifespan (~3 weeks) and two sexes: hermaphrodites (comprising most of the population) and males (comprising approximately 0.1% of the total population). After hatching, they undergo four larval stages (L1–L4) to become an adult. Under non-favorable environmental conditions, such as starvation or stress, *C. elegans* can enter an alternative third larval stage: the "dauer state". They can persist as stress-resistant dauer larvae for weeks or even months. When suitable environmental conditions are resumed, animals re-enter the life cycle at the fourth larval stage. An adult hermaphrodite produces about 300 self-fertilized eggs over a period of 3 days, and more than 1,000 eggs after male insemination.

Worms are straightforward to cultivate and propagate in the laboratory, as thousands of them can be reared on small agar-filled Petri plates or liquid media seeded with bacteria (*e.g. Escherichia coli*). In addition, worm strains can be frozen in glycerol, allowing for long-term storage. Bioinformatics and functional genomic databases providing valuable information of *C. elegans* are available on line [11], such as WormBase (http://www.wormbase.org) and WormAtlas (http://www.wormatlas.org).

One of the most remarkable features of worms is their transparent body, allowing for the visualization of all cells at all stages of development. Indeed, the complete cell lineage of *C. elegans* has been precisely described. The adult consists of 959 somatic cells, 302 of which are neurons that include chemosensory, mechanosensory, and

thermosensory types [11, 30] (Fig. **1D**). In contrast to flies, worms lack a centralized brain. Interestingly, the position of each neuron, fate and synaptic connections has been characterized in great detail [30, 31], simplifying the study of neurodegenerative phenotypes.

3.4. GENETIC APPROACHES TO STUDY NEURODEGENERATION IN FLIES AND WORMS

There are four interconnected and complementary approaches based on "reverse" and "forward" genetics to study neurodegeneration in flies and worms (Fig. **2**):

i. Transgenic models of human diseases

ii. Loss-of-function of fly or worm homologs of human disease-associated genes

iii. Screen of novel genes involved in neurodegeneration

iv. Identification of modifiers of neurodegenerative phenotypes

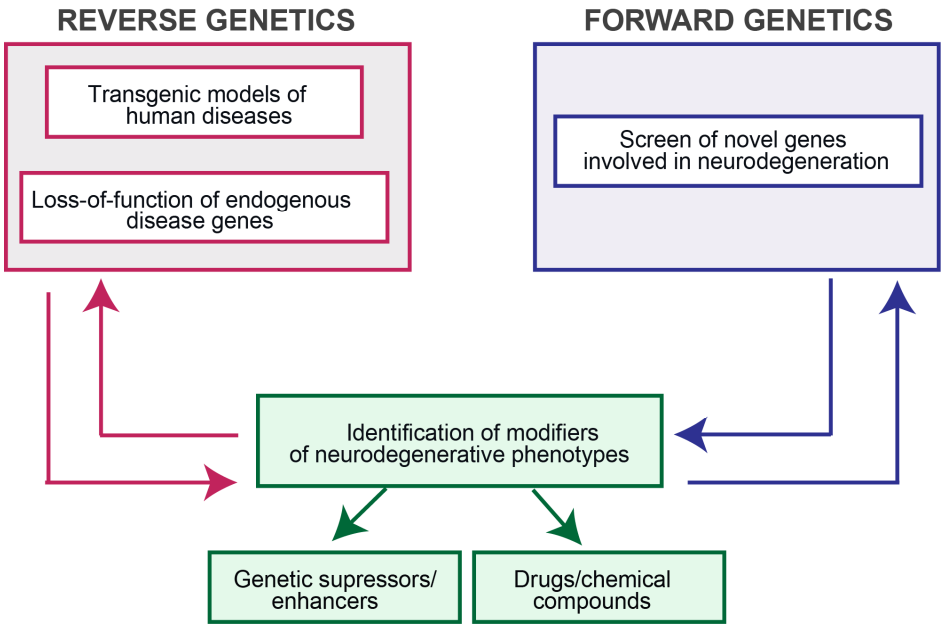

Figure 2: Using invertebrates to study neurodegeneration. The main experimental strategies employed in *D. melanogaster* and *C. elegans* to study neurodegeneration. These approaches are interconnected: a neurodegenerative phenotype caused by either (1) expression of a human disease

gene, (2) inactivation of a given disease gene ortholog or (3) mutation of a novel fly/worm neurodegenerative gene can be used as a platform to identify genetic enhancers and suppressors, or chemical compounds, that modify the original neuropathology. Genetic and chemical modifiers identified in forward genetic approaches can be studied in reverse genetic models and *vice versa.*

i) Transgenic Models of Human Diseases

"Reverse" genetics (from causal gene to phenotype) consists of evaluating the function of a candidate gene in a given biological process. Once the function of the gene has been altered, the effect on the physiology and/or behavior of the organism can be subsequently analyzed. Following such approach, wild type or mutant human genes previously linked to neurodegenerative disorders can be expressed in flies or worms. A successful transgenic model should recapitulate behavioral and pathological features of the human neurodegenerative disorder, allowing researchers to study the fundamental pathways influenced by pathological genes. By overexpressing human disease-associated genes in a specific subset of cells or tissues in both flies and worms, lethal effects resulting from broad mis-expression can be circumvented. In *Drosophila*, the GAL4/UAS binary system [32] provides a very efficient method for expressing genes in a tissue and time-dependent manner. In one parental strain, promoter regions for a particular gene drive expression of the yeast transcription factor GAL4 in defined tissues or cellular types. The second fly strain bears a transgene under the control of the upstream activation sequence (UAS) that is recognized by GAL4. The resulting progeny will express the gene of interest only in those tissues or cells expressing the GAL4 protein (Fig. **3**). In most fly disease models a human pathogenic transgene is fused to UAS (*UAS-transgene*) and expressed in a specific pattern. There are several collections of transgenic "GAL4 drivers" specific for different tissues or cell-types available to direct the expression of the gene of interest (http://flybase.org/). The eye-specific promoter GMR (Glass Multimer Reporter) has been extensively used to express pathological transgenes in the developing compound eye. This regular structure, composed of highly organized eye units known as ommatidia, allows the identification of neurodegenerative phenotypes using a standard light microscope or scanning electron microscopy to detect altered ommatidial numbers or arrangement that typically lead to a "rough" eye phenotype and loss of photoreceptor neurons (Fig. **4 A-E**). Use of GMR-based constructs has been particularly useful for genetic

enhancer/suppressor screens [33-35]. Neurodegeneration in the brain can be detected by the appearance of vacuoles (holes) (Fig. **4F,G**). Specific transgenes can be broadly expressed in the brain (*e.g. via* the pan-neuronal driver *elav-GAL4)* or, alternatively, in specific subsets of neurons (*via* neuron type-specific drivers, such as those for dopaminergic or serotonergic neurons (*TH-GAL4* or *TRH-GAL4,* respectively). Thus, it is possible to investigate cell-type-specific death associated with the expression of pathological genes. Different modifications of the GAL4/UAS system have been developed to further refine tissue specificity as well as temporal expression [36, 37]. By employing these techniques, it is possible to manipulate biological processes in the adult brain without affecting nervous system development. It should be noted, however, that high levels of GAL4 protein can trigger neuronal death *per se*, thus an excess of GAL4 might enhance neuronal defects observed in neurodegeneration models [38].

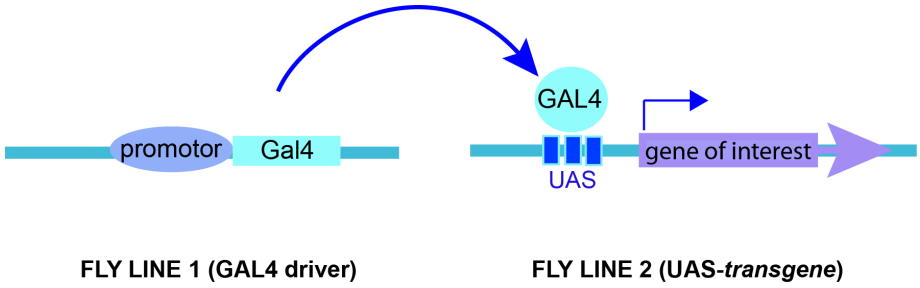

Figure 3: A powerful genetic tool to study neurodegeneration in flies. The GAL4/UAS system allows spatial and temporal gene expression in flies. In fly disease models a human pathogenic transgene is fused to a sequence (UAS) recognized by the transcription factor GAL4 (UAS-transgene). The fly line carrying such transgene is crossed to another one carrying GAL4 under the control of a specific promotor (GAL4 driver). The resulting progeny will express the gene in a specific cell or tissue type, depending on the GAL4 driver. This system also serves to knockdown the expression of an endogenous gene in selected cell types or tissues. In this case, a line expressing RNA interference (RNAi) against a specific gene under the control of UAS (*UAS-RNAi)* is crossed to the GAL4 driver line.

C. elegans can also be genetically manipulated to express human transgenes associated with neurodegenerative diseases. Generation of transgenic worms is relatively simple, low-cost and quick. It usually involves injection of transgenes into the gonad of hermaphrodite adults or bombardment with DNA-coated microparticles [39]. The *Mos*TIC technique provides a means to engineer single copy transgenes at a defined locus in the genome [40].

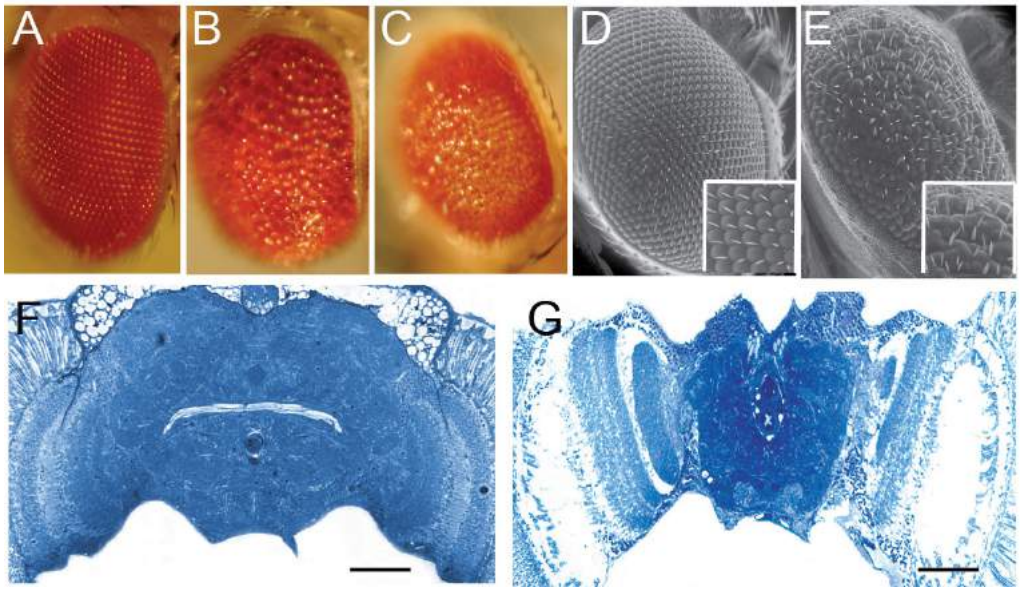

Figure 4: Neurodegenerative phenotypes in *Drosophila melanogaster*. The fly compound eye is a highly organized structure that allows detecting toxicity very easily under standard light microscopy (A-C) or scanning electron microscopy (D-E). Downregulation of the cytoskeleton gene *enabled* (*ena*) in the eye leads to disorganization of photoreceptor neurons and retinal degeneration in *GMR-GAL4>ena rev* flies (B,C,E). Images of wild-type flies showing normal eye morphology are shown in A and D. Neurodegeneration triggered by decreased *enabled* levels can be also detected in adult fly head semi-thin sections of *elav-GAL4>ena*^{rev} flies stained with methylene blue by light microscopy. Young (0–3 days) and old (30 days) *elav-GAL4>ena*^{rev} flies are shown in F and G, respectively. While the nervous system of young flies is well preserved (F), age-dependent degeneration characterized by the occurrence of vacuoles in specific areas of the brain is observed in old flies (G). Scale bar: 50 µm. Images (D-G) taken from: Rezaval *et al*., 2008. PLoS One 3, e3332.

Gene expression can be modulated by promoter-driven expression in worms; for example, transgenes can be directed to muscle cells (*via* the unc-54 promoter) or particular subsets of neurons, such as dopaminergic neurons (*via* the dopamine transporter dat-1 promoter), or touch neurons (*via* the mec-7 promoter). The transparent nature of *C. elegans* facilitates *in vivo* visualization of neurons throughout the lifetime of the animal, using fluorescent marker genes, such as the jellyfish Green Fluorescent Protein (GFP) [41, 42]. Thus, the effects of genetic and pharmacological modulators on neuronal viability can be easily evaluated in living worms by detecting signs of cell dystrophy, such as vacuolization and protein aggregation [3, 43, 44] (Fig. **5**).

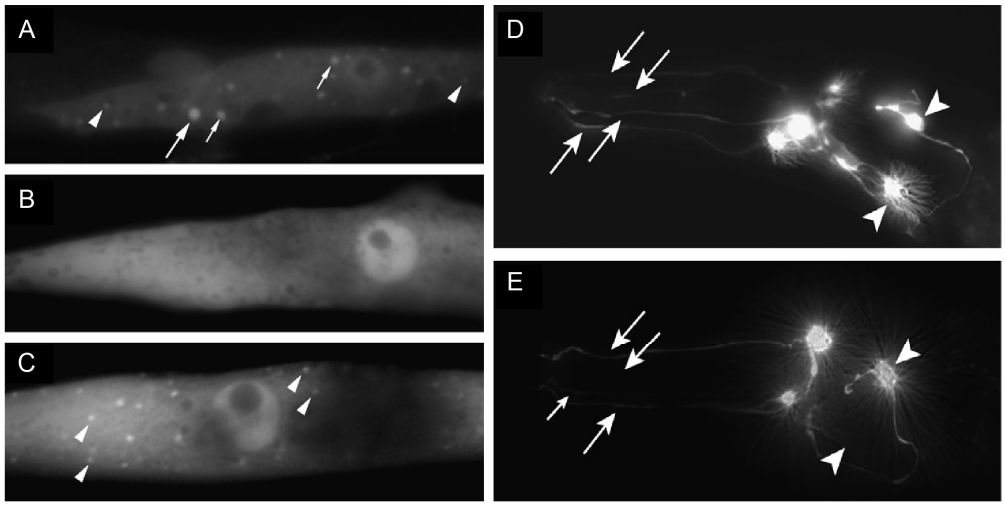

Figure 5: Neurodegenerative phenotypes in *C. elegans*. (A-C) Misfolding and aggregation of α-synuclein in nematodes. Photomicrographs of transgenic *C. elegans* displaying muscle-specific expression of α-synuclein: GFP. (A) Misfolded α-synuclein forms small (arrowhead), medium (small arrow) and large aggregates (arrow) in body wall muscle cells. (B) Decreased α- synuclein: GFP misfolding is observed when the chaperone TOR-2 is simultaneously expressed, demonstrating the utility of this transgenic strain in screening for enhancers/suppressors of misfolding. (C) Following RNAi of specific gene targets in worms expressing TOR-2 + α synuclein: GFP misfolded α-synuclein is again detected (arrows). (D-E) α-synuclein-induced toxicity in dopaminergic neurons in *C.elegans*. (D) The six dopaminergic neurons in the anterior region of *C. elegans* (four CEP and two ADE neurons) are visualized in a transgenic Pdat-1: GFP worm where GFP is under the control of the dopamine transporter promoter. The dendrites of the CEPs are indicated with large arrows and the cell bodies of the ADEs are shown with arrowheads. (E) CEP and ADE age-dependent degeneration is observed in worms expressing both Pdat-1: GFP and Pdat-1: α-synuclein. A 7-day old worm displays two intact CEP neurons (large arrows), one retracting (degenerating) CEP dendrite (small arrow), and one intact ADE neuron (arrowhead). The additional CEP and ADE neurons have degenerated and are no longer visible (grey arrow and arrowhead represent normal locations of the CEP and ADE, respectively). Images taken from: Harrington *et al*., 2011. Methods 53, 220-225.

ii) Loss-of-Function of fly or Worm Homologs of Human Disease Genes

Following a reverse genetic approach, fly or worm genes with similarity to specific human familial neurodegenerative disease-associated genes can be disrupted and the resulting mutant phenotype investigated. In *Drosophila*, total or partial genetic inactivation can be achieved *via* (1) transposon-mediated mutagenesis [9, 45-49], (2) GAL4/UAS–mediated RNA interference (RNAi) [48, 50] and (3) homologous recombination-based gene knockout [51-53]. Several large-scale P element gene disruption projects have generated thousands of stocks

of flies containing single P elements insertions at known locations in the genome. Many of these are available in fly stock centers (http://www.flybase.org). P elements have become key genetic tools in *Drosophila*, used not only as mutagens but also for *in vivo* gene tagging and inserting transgenes [9, 48, 49].

The second mutational strategy is based on RNA interference (RNAi), the gene-specific degradation or inhibition of an mRNA that prevents the encoded protein from being synthesized. This evolutionarily conserved mechanism was first characterized in *C. elegans*, and is triggered by double stranded RNA (dsRNA) that shares sequence identity to a specific mRNA [54]. RNAi is cleaved *in vivo* into short fragments that guide sequence-specific mRNA degradation or translational repression. The technique of RNAi, coupled with the availability of the complete genomic sequences of *Drosophila* and *C. elegans* has made possible the rapid study of gene function, both on a single gene level and at a global scale. In flies, specific RNAi targeting can be achieved *via* the GAL4/UAS system: a UAS line expressing an RNAi sequence (*UAS-RNAi*) is used in conjunction with a specific GAL4 line to knockdown the expression of the gene in selected cell types or tissues. Collections of RNAi knockdown strains targeting ~90% of the entire *Drosophila melanogaster* genome are available to the research community [50]. It should be noted, however, that RNAi often results in only partial gene inactivation or, in some cases, no inactivation at all. In this regard, the use of the enzyme Dicer has greatly improved the efficiency of the RNAi methodology in *Drosophila*, such that dsRNAs are better processed in the presence of this enzyme [55].

In the third mutational strategy, homologous recombination-based gene knockout allows precise gene targeting to eliminate a specific gene [51, 52, 56]. Gene targeting is the modification of an endogenous gene sequence by recombination between an introduced DNA fragment and the homologous target gene [57]. This method has proven to be a valuable tool for altering genes in mice [58] as well in *Drosophila* [51, 56].

Currently, several methods are available for genetic inactivation of genes in *C. elegans,* including i) transposon-mediated mutagenesis and ii) use of RNAi [54, 59]. As in *Drosophila*, transposon-mediated mutagenesis in *C. elegans* is based on active transposable elements to inactivate gene function. In such

approaches, transposons are mobilized randomly in a *Drosophila* or *C. elegans* strain and independent lines are then screened for the presence of a transposon insertion in the gene of interest [60, 61].

RNAi-based approaches to inactivate gene function have been utilized extensively in worms [54, 59]. Specific dsRNA can be delivered into the worm either by microinjection, at the single worm level, or by uptake of dsRNA by worms in solution (soaking) or feeding worms bacteria engineered to synthesize the specific dsRNA. The most reliable method for causing severe gene inhibition is microinjection; however, it is more labor intensive than other approaches. Soaking and feeding are more suitable methods for high-throughput genomic analyses, as large numbers of worms can be treated at once. Since *C. elegans* neurons are refractive to RNAi and not efficiently targeted by bacterial feeding approaches [59], techniques that aim to improve RNAi efficiency in neurons have been developed, such as selecting mutant strains that are more sensitive to RNAi [62, 63]. However, it should be noted that strains with intrinsic mutant phenotypes could also interfere with the process of interest.

More recently, homologous recombination methods have been developed to specifically disrupt gene function [64]. Yet these approaches are still laborious and not amenable for large scaling.

iii) Screen of Novel Genes Involved in Neurodegeneration

Classical “forward genetic” screens (from phenotype to causal gene) involve the generation of random mutations in the genome, screening of the resulting mutants for a specific phenotype and subsequent identification of the affected gene. Compared to “reverse genetics”, this approach is an unbiased method as it does not require previous knowledge about the nature of the emerging candidates. Therefore, unexpected genes and molecular pathways involved in a disease pathogenesis can be uncovered, which can be further studied in more complex animals. Large collections of mutants can be anatomically analyzed in search for “neurodegeneration hallmarks”, such as abnormal accumulation of pathogenic proteins and age-dependent neuronal death that can be identified by direct examination of the brain. Genetic screens can also be based on the observation that neurodegeneration is often associated with neuronal dysfunction that results

in altered physiology or behavior. Hence, these approaches select for mutations causing neuronal death, reduced lifespan or abnormal behaviors, such as progressive incoordination or paralysis [24].

How difficult is to produce *Drosophila* mutations in a large-scale? Ionizing radiation and chemical mutagens, such as ethyl methane sulphonate (EMS) or N-ethyl-N-nitrosourea (ENU) are used to produce mutations in *Drosophila*. However, the identification of the resulting mutations at the DNA level is labor intensive, time consuming and consequently not practical on a genome-wide basis. In contrast, P element-induced mutations can be rapidly identified on a large scale. However, P elements tend to integrate preferentially into specific hotspots, thus reducing the proportion of the genome that can be randomly targeted. Several genome-wide collections of chemical or transposon-induced mutants are currently available from *Drosophila* stock centers. Alternatively, large collections of transgenic RNAi strains are also available and can be employed in combination with GAL4 lines to screen for mutant phenotypes [50, 65]. The RNAi technique overcomes a clear limitation of traditional chemical or radiation mutagenesis screens in that it allows the identification of genes that generate lethality earlier in development, through restricting gene inactivation in a temporal and/or spatial fashion. In addition, the gene responsible for the phenotype of interest is already known, making it possible to establish a connection between a phenotype and the affected gene rapidly.

In *C. elegans*, large mutant libraries are obtained by either (1) chemical mutagenesis, such as EMS, diethyl sulfate (DES) or N-nitroso-N-ethylurea (ENU); (2) irradiation with X-rays, γ-rays or UV light; (3) transposable element movement. Since chemical mutagens are easy to use, efficient and create a wide range of genetic lesions, they have been successfully used to generate mutant libraries for PCR-based identification of deletions in genes of interest [66]. The *C. elegans* Gene Knockout Consortium (GKC: http://celeganskoconsortium.omrf.org/) and the National Bioresource Project (NBRP: http://www.shigen.nig.ac.jp/c.elegans/index.jsp) have been established to isolate deletion mutants for all *C. elegans* genes. As in *Drosophila*, a critical bottleneck for chemical mutagenesis resides in the arduous task of genetic mapping and identification of the mutant gene. Transposon-based insertional mutagenesis is a strategy complementary to chemical mutagenesis and

greatly facilitates identification of mutant genes. However, the identification of mutagenic insertions can be complicated by the presence of multiple endogenous transposons in the worm genome. In this regard, mobilization of heterologous transposons such as the *Drosophila* mariner transposon *Mos1,* has provided a more efficient way to generate mutations. These mariner elements constitute unique transposon insertions in a *C. elegans* mutant strain and are thus more readily localized [67]. Unfortunately, such transposons are not as versatile as P elements in *Drosophila* and are not useful for introducing large DNA fragments into the worm genome. The fact that there is also a bias in the sites of mariner insertions in the worm's genome represents an additional caveat. In this regard, the NemaGENETAG project (http://elegans.imbb.forth.gr/nemagenetag/) is contributing to expand the library containing transposon-tagged mutants in *C. elegans*. Emerging technologies, such as Mos TIC approach for genome engineering, allow the introduction of exogenous DNA sequences into predetermined genomic locations [40, 68]. Alternative approaches for gene inactivation include the use of RNAi in worms (*e.g.* [69]). RNAi by feeding is the least labor intensive and the most economical method used for high throughput genome-wide screenings, allowing large numbers of genes to be evaluated simultaneously [70]. Lethal phenotypes at the embryonic stage can be avoided by delivering dsRNA into the worms at the first larval stage. The use of RNAi libraries has been very effective in screening for a variety of worm phenotypes/genes [70]. There are currently two RNAi feeding libraries for *C. elegans*: known as the Ahringer and ORFeome libraries. These libraries are available to the public and together, can target about 94% of *C. elegans* genes. Importantly, WormBase contains valuable information about published large-scale RNAi data, including genes affected and all RNAi phenotypes.

iv) Identification of Modifiers of Neurodegenerative Phenotypes

Once a neurodegenerative phenotype caused by, for example, expression of a human disease-associated gene or inactivation of a given disease -associated gene ortholog is established, it is possible to perform genetic modifier screens to identify genetic enhancers and suppressors that modify the original neuropathology. In addition, pharmacological screens can be used to identify drugs that modulate the neurodegenerative phenotype and their associated molecular pathways. Therapeutic compounds that have been already identified in mammalian systems can also be

tested in invertebrate models, allowing for validation of hits and exclusion of compounds with unfavorable properties [71-73].

Moderate ‘‘high-throughput’’ screens are possible in *Drosophila* by feeding flies with food mixed with different concentrations of neuroactive compounds. The lack of a stringent blood-brain barrier in flies simplifies the access of pharmacological compounds to the nervous system. An alternative approach to deliver pharmacological compounds in a more efficient way consists of direct intra-abdominal or intra-thoracic injection into adults. However, this method is more laborious and time-consuming [74]. In this regard, microfluidic devices in combination with computer-controlled injection systems provide a more systematic methodology for injections in fly embryos [75, 76].

C. elegans also lacks a functional blood-brain barrier and are sensitive to a wide range of human neuroactive drugs [77]. Worms can be grown in liquid medium in 96-well microtiter plates containing different drugs and rapidly screened for novel compounds. Thus nematodes are even more amenable for large therapeutic screens than *Drosophila* [77]. Neuronal viability can be measured with GFP-based tags in a fluorescent plate reader or, alternatively, worm motor activity can be automatically monitored in real time [78]. Both these approaches are amenable to high throughput screens and allow the identification of neurotoxic or neuroprotective compounds.

Once a therapeutic drug has been identified in either *Drosophila* or *C. elegans* model systems, suppressor screens can be carried out to identify molecular partners involved in the compound-mediated protection of neurons. Drugs with efficacy in invertebrate models, however, need to be validated in mammalian whole-animal disease models to be considered as candidates for clinical trials [73].

3.5. CONTRIBUTIONS OF FLY AND WORM STUDIES TO UNDERSTANDING THE MECHANISMS UNDERLYING NEURODEGENERATION

3.5.1. Insights from Transgenic and Mutant Models of Human Diseases

Examples of findings associated with pathogenic mechanisms and therapeutic implications in flies and worms are offered here to illustrate the value of invertebrate models in the study of neurodegeneration.

3.5.1.1. Alzheimer's Disease

Patients with Alzheimer's disease suffer from age-dependent memory loss, deterioration of cognitive functions and dementia. Progressive neuronal degeneration affects specific areas of the brain, such as the frontal cerebral cortex and hippocampus [79]. The pathological hallmarks of Alzheimer's disease are the accumulation of extracellular senile plaques (composed mainly of the amyloid Aβ42 peptide) and intracellular neurofibrillary tangles (composed of aggregated, hyperphosphorylated forms of the microtubule-associated protein Tau) [79]. Aβ42 peptides are produced by proteolytic cleavage of the amyloid precursor protein (APP) transmembrane receptor *via* the action of β and χ-secretases. The ß-site APP-cleaving enzyme (BACE) cleaves APP at the beta site, and the presenilins, PS1 and PS2, participate in APP cleavage at the ɣ site [80]. Genetic analyses of familial Alzheimer's disease identified mutations in the APP, PS1 and PS2 genes; these mutations are all associated with abnormal APP processing and Aβ42 aggregation. Hence, it is believed that Aß42 peptide overproduction is the initial trigger of a series of pathogenic events that result in Tau hyperphosphorylation, abnormal cellular signaling, and synaptic failure, ultimately leading to neuronal death [81-83].

Different approaches have been utilized to study the normal function of APP, as well as the mechanisms by which APP dysfunction might lead to neurodegeneration in flies [4, 72]. Flies carrying loss-of-function mutations of the fly homolog of the human APP (dAPPL) display no neurodegeneration but show abnormal behaviors that are rescued by the transgenic introduction of human APP gene, indicating functional conservation between fly APPL and human APP [84]. Additional studies have linked dAPPL with physiological functions such as neuronal development and synapse formation [85-87]. Transgenic flies expressing wild type and Alzheimer's disease mutant forms of APP in the fly nervous system and retina have revealed interesting findings. APP and dAPPL overexpression leads to axonal transport defects [85, 88] that appear to correlate with impaired synaptic plasticity [89]. Evidence for dysfunction in axonal transport has also been found in other neurodegenerative disease fly models, such as Huntington's disease [90, 91], suggesting a common mechanism of neurodegeneration in different diseases.

Flies have orthologs of α-secretases [92, 93] and γ-secretase components, such as PSN and Nicastrin [94-96]. Although dAPPL lacks the Aβ domain [96b], it has been recently shown that processing of dAPPL by the fly β-secretase BACE (dBACE) results in neurotoxic Aβ-like fragments, amyloid deposits and neurodegeneration [97]. Aβ42 formation and neurotoxicity can be achieved by simultaneous expression of human BACE and APP in either the fly retina or nervous system [98, 99]. Interestingly, direct expression of human Aβ42 in the fly recapitulates aspects of the Alzheimer's disease pathology, such as age-dependent neurodegeneration and accumulation of amyloid plaques [100, 101]. Moreover, generation of Aβ42 leads to defective axonal transport [85, 88], mitochondrial mislocalisation [102] and synaptic plasticity defects [89]. Aβ42 accumulation has been also found to trigger progressive locomotor deficits, abnormal learning and reduced lifespan [99, 100, 103, 104]. On the other hand, different studies in flies have confirmed experimentally that Aß42 aggregation propensity correlates with neurotoxicity [72, 99, 105, 106] and several modifiers of Aβ42 aggregation-related toxicity have been identified. Some of these include genetic regulators of proteolytic processing of APP, such as modulators of PSN activity [107, 108] or compounds that interact with amyloid structure and reduce aggregation properties of Aβ [103, 109]. These findings highlight the utility of *Drosophila* models in providing indications of pathogenic mechanisms and identifying Alzheimer's disease compounds that target Aß42 aggregation to reduce toxicity.

As in humans, several studies in *Drosophila* have implicated heavy metals in the development of Aβ-induced pathological processes [110]. One study shows that inhibition of zinc transporters reduces Zn^{++} accumulation in the fly brain, which in turn reduces Aβ42 deposits [111]. Thus, manipulation of zinc transporters in Alzheimer's disease brains may represent a novel therapeutic strategy.

C. elegans contains one APP-related gene (*apl-1*) that similarly to dAPPL, lacks a region equivalent to the Aβ-peptide [112]. Knockout of *apl-1* causes developmental defects and larval lethality while overexpression produces movement defects and reduces viability. Interestingly, RNAi knockdown studies revealed a role for apl-1 in synaptic transmission [113]. Similarly to *Drosophila,* Aβ42 peptides have been expressed in *C. elegans* to study different aspects of Alzheimer's disease. Thus, worms carrying transgenes that drive Aβ42 peptide

expression in body wall muscles or neurons have been created [114, 115]. Aβ42 expression under the control of a muscle-specific promoter leads to intracellular amyloid deposits in the muscles, in addition to progressive paralysis and reduced lifespan in worms [44, 114-116]. This model has been subsequently used to identify binding partners of Aβ that contribute or respond to Aβ toxicity [117-119]. Interestingly, Aβ deposits induce stress response in worms by promoting expression of heat shock proteins. Thus, heat shock chaperone function might play a role in modulating intracellular Aβ42 metabolism and toxicity [116-118]. Aβ42 accumulation has been also found to induce increased iron levels and oxidative stress in human cell and worm models of Alzheimer's disease [120, 121]. Carbonyl accumulation, an oxidative damage indicator, also correlates with Aβ42 expression in worms. This phenomenon has also been observed in human neuronal cultures exposed to Aβ42 and brain tissue from Alzheimer's disease patients [122]. Aβ-expressing nematodes have also served to identify potential therapeutic reagents of the Aβ42-related toxicity [23]. Tetracyclines have been found to interact with Aβ42 oligomers and prevent their aggregation in Aβ transgenic worms [123]. Moreover, these compounds decrease superoxide production, and thus oxidative stress, in Alzheimer's disease nematode models. These findings suggest a potential use of these drugs for reducing Aβ aggregates. Additional compounds useful for preventing Aβ42 toxicity include coffee extracts [124] and extracts from the *Ginkgo biloba* that have been shown to decrease reactive oxygen species (ROS) generated by oxidative stress [125, 126].

In alternative approaches, *Drosophila* and *C. elegans* models for tauopathy have been established to study the pathological properties of intracellular neurofibrillary tangles and Tau associated with Alzheimer's disease. These strategies have been recently reviewed and therefore will not be discussed here [3, 21, 23, 127, 128].

3.5.1.2. Parkinson's Disease

Parkinson's disease is characterized by age-dependent loss of dopaminergic neurons in the brain, resulting in loss of motor capacity, involving tremors, rigidity and bradykinesia, as well as cognitive disorders [129]. The progressive degeneration of dopamine neurons has been associated with the formation of inclusion bodies called Lewy bodies, containing misfolded and aggregated α-synuclein protein [130, 131].

Familial forms of Parkinson's disease have been linked to mutations in *α-synuclein, ubiquitin carboxy-terminal hydrolase-L1* and *Parkin* genes. These findings implicate Lewy body components and defects in the degradation of misfolded/aggregated proteins in the mechanism of the pathology [131, 132].

Expression of wild type and mutant forms of human *α-synuclein* in flies recapitulate key features of Parkinson's disease such as inclusions of α-synuclein reminiscent of Lewy bodies, age-dependent degeneration of dopaminergic neurons and progressive motor [133-137]. Expression of Hsp70, a highly conserved molecular chaperone involved in refolding of misfolded proteins, can prevent the pathology of α-synuclein Parkinson's disease models in flies [134] and mice [138]. Moreover, pharmacological treatments involving geldanamycin suppress α-synuclein toxicity in flies by inducing the heat shock response [139], suggesting a potential therapy for Parkinson's disease. Notably, human genetic data shows that some polymorphisms in Hsp70 are genetic risk factors for Parkinson's disease [140]. These findings reveal a role for abnormal protein folding and aggregation in the disease pathogenesis [134, 141]. Interestingly, superoxide dismutase activity prevents the death of dopaminergic neurons in flies, highlighting the importance of oxidative stress in the α-synuclein pathogenesis [142]. In addition to abnormal protein aggregation and oxidative damage, altered histone acetylation is involved in Parkinson's pathogenesis. α-synuclein inhibits histone acetylation in the nucleus and induces neurotoxicity that can be reverted by histone deacetylase inhibitors [143]. Other studies have focused on the role of phosphorylation in the generation of neurotoxic isoforms of α-synuclein [144-147], providing new insight into the signaling pathways underlying Parkinson's disease.

Several genes have been associated with autosomal recessive juvenile parkinsonism, including *DJ-1*, *Pink1* and *Parkin*. *Drosophila* homologs of these genes exist and have been mutated. These mutants are associated with mitochondrial disruptions [148], supporting the notion that mitochondrial dysfunction is an important factor underlying the pathogenesis [4]. The *Parkin* gene, an E3 ubiquitin protein ligase, is involved in proteasomal degradation of damaged proteins. *Drosophila PARK2* null mutants exhibit increased oxidative stress, reduced lifespan, behavioral defects and age-dependent muscle degeneration associated with neuronal apoptosis and mitochondrial pathology [149-151]. Other studies suggest that PINK1 and Parkin act

together in a common pathway regulating mitochondrial morphology and function, including mitochondrial fission/fusion [152-156]. New *Drosophila* models of mitochondrial dysfunction are emerging to specifically study the mechanisms responsible for mitochondria-mediated dopaminergic neuronal loss [157]. Biochemical approaches have revealed genetics modulators of Parkin-mediated toxicity. For example, glutathione S-transferase (GST) S1 activity is sufficient to rescue dopaminergic neuronal death in Parkin mutants, likely by modulating cellular response to oxidative stress [151, 158, 159]. In addition, another member of the GST family in *Drosophila* (glutathione S-transferase Omega 1) suppresses the phenotypes of *parkin* and *α-synuclein* mutants by regulating mitochondrial ATP synthase activity [160].

Environmental toxins, such as the herbicide paraquat and the pesticide rotenone, appear to be risk factors for sporadic Parkinson's disease. Exposure of flies to such environmental contaminants leads to increased oxidative stress through mitochondrial pathways. These treatments induce parkinsonian-like symptoms in *Drosophila*, including dopaminergic neuronal death and behavioral abnormalities that can be mitigated by adding the antioxidant melatonin. These findings suggest that antioxidants may be helpful in the treatment of the Parkinson's disease pathology [161, 162].

C. elegans models of Parkinson's disease have also been generated that examine α-synuclein toxicity. α-synuclein::GFP or α-synuclein::YFP (Yellow Fluorescent Protein) fusion proteins were expressed specifically in either body-wall muscles, the nervous system or specific subsets of neurons, such as motor neurons or dopaminergic neurons. Overexpression of wild type and mutant forms of human α-synuclein in dopaminergic neurons has been shown to trigger neuronal loss accompanied by accumulation of misfolded α-synuclein aggregates in *C. elegans* [163, 164]. Since nematodes have only eight dopaminergic neurons, it is straightfoward to examine their integrity over time. Interestingly, α-synuclein overexpression renders these worms incapable of reducing their locomotion upon the presence of food, a behavioral response controlled by dopaminergic neurons. This phenotype can be rescued by re-establishing normal dopamine levels [164]. High throughput genomic approaches using α-synuclein transgenic worms have uncovered changes in expression of genes associated with components of

ubiquitin-proteasomal and mitochondrial systems that may arise as a consequence of mitochondrial dysfunction in the Parkinson's pathology [165].

Large-scale RNAi screens in worms have permitted identification of modulators of the neurodegenerative phenotype triggered by α-synuclein expression *via* the premature formation of aggregates of fluorescently labelled misfolded proteins *in vivo* [166]. Other studies have identified modifier genes associated with protein degradation, lipid metabolism, RNA metabolism, vesicular trafficking and endocytosis pathways, in addition to aging-associated genes [166-172]. Several of these modifiers have been successfully validated as neuroprotective in mammalian systems. An interesting example is VPS-41, a protein involved in lysosomal trafficking of Golgi-derived vesicles [173]. The human ortholog of VPS-41 (hVPS41) also protects *C. elegans* neurons and mammalian neuroblastoma cells from the toxic effects produced by Parkinson's disease-associated toxins [174]. Recent studies suggest that hVPS41 prevents α-synuclein toxicity by facilitating clearance of misfolded and aggregated proteins [175].

MicroRNAs (miRNAs) are short ribonucleic acid (RNA) molecules that repress mRNA translation or mediate mRNA degradation in a sequence-specific manner in animals and plants [176]. Interestingly, microRNA regulation has been linked to Parkinson's pathogenic mechanisms in disease worm models, as is the case in mammalian systems, suggesting a conserved pathological mechanism across species [177].

C. elegans have orthologs for various human genes linked to familial Parkinson's disease, including parkin (pdr-1), PINK1 (pink-1) and DJ-1 (djr-1.1, 1.2). Mutations in these genes can cause loss of dopaminergic neurons or mitochondrial pathology [178, 179]. pdr-1 null mutants exhibit lower levels of ubiquitin conjugates, suggesting that alterations in the ubiquitin proteasome system may be a causative factor for the pathogenesis of Parkinson's disease [178].

In addition to genetic models of Parkinson's pathology in worms, the effects of environmental agents have also been evaluated. For example, worms exposed to either 6-OHDA or rotenone (neurotoxins with harmful effects in rodents [180]), show selective degeneration of dopaminergic neurons [167, 181, 182]. Studies

using these models demonstrate that restricted diet can prevent dopaminergic neuron degeneration [183, 184], suggesting a link between Parkinson's and metabolism. Neurotoxicity models of dopaminergic neuron degeneration have also revealed oxidative stress and the protein misfolding are major contributing factors in neurodegeneration and disease progression [183, 185].

3.5.2. Insights from Neurodegenerative Mutations

Forward genetic screens have identified a number of interesting mutations that cause neuronal dysfunction and death. This strategy has been enormously successful in flies. Pioneering studies in neurodegeneration were carried out in Seymour Benzer's laboratory, one of the most influential laboratories in the history of *Drosophila* neurogenetics. Fly mutants such as *bubblegum*, *swiss cheese* and *drop-dead* were first isolated in screens selecting for flies with defective phototaxis behavior or reduced lifespan, followed by histological examination that revealed vacuolization in the brain [186-189]. Bubblegum is considered an interesting candidate to study "human-like neurodegeneration processes". It has a mutation in the *VLCFA acyl coenzyme A synthetase* gene that leads to abnormal accumulation of very long chain fatty acids, as it is observed in patients with adrenoleukodystrophy (ALD). Moreover, such neurodegenerative phenotype can be alleviated by feeding the flies with 'Lorenzo's oil', a treatment based on monounsaturated fatty acids used to lower VLCFA levels in ALD patients [189]. Other studies have isolated fly mutants on the basis of additional neurodegenerative phenotypes, including paralysis induced by high temperature or mechanical stress [190, 191] and abnormal circadian rhythms [192]. These and additional large-scale genetic screens have identified mutations that interfere with mitochondrial function, signal transduction, lipid homeostasis, protein homeostasis, channel function, cytoskeleton, oxidative stress response and glial-neuronal signaling in *Drosophila* [24, 28]. The characterization of these mutants has shown that many of them recapitulate important features of human neurodegenerative diseases, *i.e.*, vulnerability of specific neuronal populations and progressive degeneration. Interestingly, the importance of some of these genes in neurodegeneration has been validated in mammalian disease models (*e.g.*[193, 194]). This suggests that this approach may identify novel genes important for conserved mechanisms that maintain nervous system integrity.

CONCLUDING REMARKS

The generation of animal models that recapitulate physiologic and pathologic conditions in humans are key for biomedical and scientific progress. They provide an opportunity to explore the mechanisms underlying disease pathogenesis as well as develop effective preventive measures and therapies. During the past decades, invertebrate models of neurodegeneration have emerged as successful complementary systems to mammalian models, facilitating identification of relevant pathways and novel disease-associated genes. It is important to bear in mind, however, that invertebrate models have potential caveats and limitations in studying the function of human disease genes. They lack a number of disease-related factors and biophysical processes that may influence specific pathologies. It follows that observations in invertebrates should be subsequently validated in mammals to determine their relevance to human diseases. Nevertheless, the conservation of important basic biological processes in *Drosophila, C. elegans* and mammals have permitted the recreation of essential pathological features observed in human patients, substantiating their enormous potential for dissecting conserved pathogenetic mechanisms [3, 4, 20-26]. Moreover, these simple models offer extraordinary genetic tools to decipher genetic pathways of disease-genes and to discover genetic factors that modulate the neurodegenerative phenotype. Notably, findings from worm and fly models of Aβ42 toxicity, polyglutamine repeat proteins and α-synuclein have identified conserved chaperone proteins as important suppressors of neurotoxicity [117, 118, 134, 195]. These findings suggest that some toxic mechanisms might be common to different neurodegenerative diseases. The fact that overexpression of *glutathione-S-transferase* can suppress the toxicity associated with either long polyglutamine repeat proteins, *α-synuclein* overexpression or mutations in *Parkin* also implicates oxidative stress as playing a role in different neurodegenerative processes [3, 4, 151, 158-160]. Importantly, several genetic modulators identified in enhancer/suppressor screens have been validated in mammalian systems [174, 175, 196-200]. In addition, loss-of-function studies of endogenous genes homologous to human disease genes in *Drosophila and C. elegans* have yielded new clues to pathogenic mechanisms. For example, mitochondrial dysfunction was first linked to defective PINK1/Parkin signalling in *Drosophila* [152, 153, 156]. On the other hand, invertebrate neurodegenerative

mutants isolated in forward screens that show characteristic features of progressive neurodegeneration have provided valuable insights into conserved genetic pathways and mechanisms required for maintaining the structural integrity of the nervous system. Indeed, this approach has allowed researchers to identify novel neurodegeneration genes, suggesting their human orthologs may also be involved in pathological processes [24, 28]. Since collections of genetic mutations in flies and worms are constantly expanding, it is reasonable to anticipate that additional disease genes will be identified.

Pharmacological screens in worms and flies have identified potential therapeutic compounds. For example, the identification of chaperones and histone deacetylase inhibitors as suppressors of neurodegenerative phenotypes in flies has led, in some cases, to validation in mouse models and human clinical trials [34, 35, 72, 134, 139, 141]. Sophisticated and automated techniques that are impractical in mammals continue to emerge in invertebrates. These will increasingly facilitate high-throughput screens for candidate therapeutic reagents.

Given the rapid advances in the field of neurodegeneration in *Drosophila* and *C. elegans,* it is logical to expect an increasing number of high-quality studies that will continue to enrich the study of neurodegenerative diseases and complement studies in mammalian systems. Futures studies in invertebrates will focus on understanding key aspects of the neurodegenerative pathology, including ageing and disease susceptibility. Illumination of these processes is essential for diminishing events that promote age-associated neuronal decline and disease. Such studies may ultimately provide a molecular link between ageing and neurodegeneration.

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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Section 2: Cellular Mechanism for Neurodegeneration

CHAPTER 4

Calcium in Homeostasis and Neurodegeneration

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Abstract: All brain functions are controlled by specific synapses where the release of neurotransmitters triggers a number of signaling cascades in postsynaptic neurons. One of the most important and common events is a transient and very fast intracellular Ca^{2+} increase. Intracellular Ca^{2+} increase is fundamental for modulation of gene expression, neuronal survival and plasticity. In this chapter we will discuss the importance of Ca^{2+} in cells as well as the regulation of physiological functions in various organisms. Additionally, we will consider mechanisms used by the cells for Ca^{2+} homeostasis and for increasing intracellular Ca^{2+} concentration. Finally, the role of Ca^{2+} in neurodegenerative diseases such as Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), Huntington disease (HD) among others will be discussed in this chapter.

Keywords: Calcium, Alzheimer's disease, Parkinson's disease, Cellular signaling, neurodegeneration, neurodegenerative diseases, brain, Inositol Trisphosphate, Endoplasmic Reticulum, Voltage gated Ca^{2+} channels, glutamate receptors, ATP receptors, Amyotrophic Lateral Sclerosis, Huntington's Disease, Multiple Sclerosis, SERCA pump, Ligand Ca^{2+} channels, Calcium Dysregulation, Ca^{2+}-ATPase, Plasma membrane.

4.1. CALCIUM

During the evolution of organisms, several molecules, proteins and ions emerged as fundamental signaling agents responsible for regulating essential functions in cells, organs and the whole organism. Among these agents, the calcium ion (Ca^{2+}) is the most versatile as it plays a key role in several aspects of cellular physiology.

Calcium is the most abundant ion in vertebrates (around 30g per kg of body weight in humans and other vertebrates). Although calcium is mostly found in

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teeth and bones in the form of calcium carbonate [1], Ca^{2+} plays a pivotal role in physiological and biochemical processes of the organisms and cells.

Both extracellular and intracellular calcium have fundamental roles in several signaling and physiological functions. The elevated extracellular free calcium concentration (mM), compared to the low intracellular free calcium concentration (nM), results in a Ca^{2+} electrochemical potential gradient. Minimal changes in these concentrations, induced by physiological stimuli that alter the permeability of the plasma membrane to these ions, can induce significant fluctuations in the levels of cytosolic Ca^{2+} thereby triggering the activation or inhibition of several physiological processes. Ca^{2+} ions play an important role in several biological functions in eukaryotic cells - organization of the cytoskeleton, cellular division and differentiation [2-5], opening and closure of stomata guard cells [6], modulation of neurotransmitter release from neurons [7], contraction of muscle cells [8], and cell death [9], among others (Fig. **1**). Moreover, Ca^{2+} can bind to several proteins including calbindin, calmodulin and proteins of the S100 family, which regulate Ca^{2+}-dependent metabolic processes. Although Ca^{2+} plays an important role in regulating many cellular processes, high concentrations of Ca^{2+} in the cytosol can be cytotoxic because they promote cellular damage and cell death. Due to the fact that many cellular processes, ranging from cellular division and differentiation to cellular death, are modulated by an increase in intracellular Ca^{2+} concentration, these levels are tightly regulated *via* many mechanisms present in the cells.

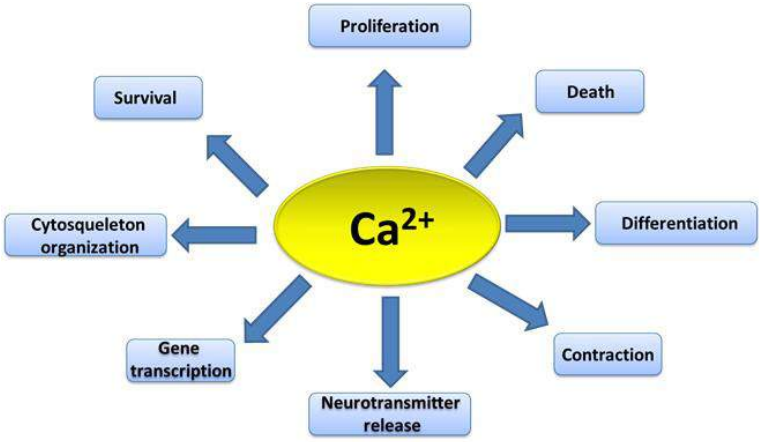

Figure 1: Physiological roles of Ca^{2+}

4.2. MECHANISMS UTILIZED BY EUKARYOTIC CELLS TO MAINTAIN LOW LEVELS OF CYTOSOLIC CA^{2+}

Eukaryotic cells employ several mechanisms to regulate and maintain cytosolic Ca^{2+} concentration at low levels (Fig. **2**). These include Ca^{2+} transport across the plasma membrane through several proteins including the Ca^{2+}/Na^{+} exchanger as well as the accumulation of Ca^{2+} in the endoplasmic reticulum, mitochondria and acidic pools. Additionally, there are Ca^{2+}-ATPases, which utilize ATP as an energy source to transport Ca^{2+} from the cytosol to either the extracellular medium across the plasma membrane (PMCA - Plasma Membrane Ca^{2+}-ATPase) or to the endoplasmic reticulum (SERCA - Sarco Endoplasmic Reticulum Ca^{2+}-ATPase). In the mitochondria, Ca^{2+} uptake depends on an electrochemical gradient generated at the inner mitochondrial membrane and ATP hydrolysis is not required. In addition, there are some proteins that can bind Ca^{2+} in the cytosol and thereby act as a Ca^{2+} buffer (*i.e.* Calmodulin). Finally, some organelles which have an acidic lumen (acidic calcium pools) can also be involved in calcium uptake from the cytosol.

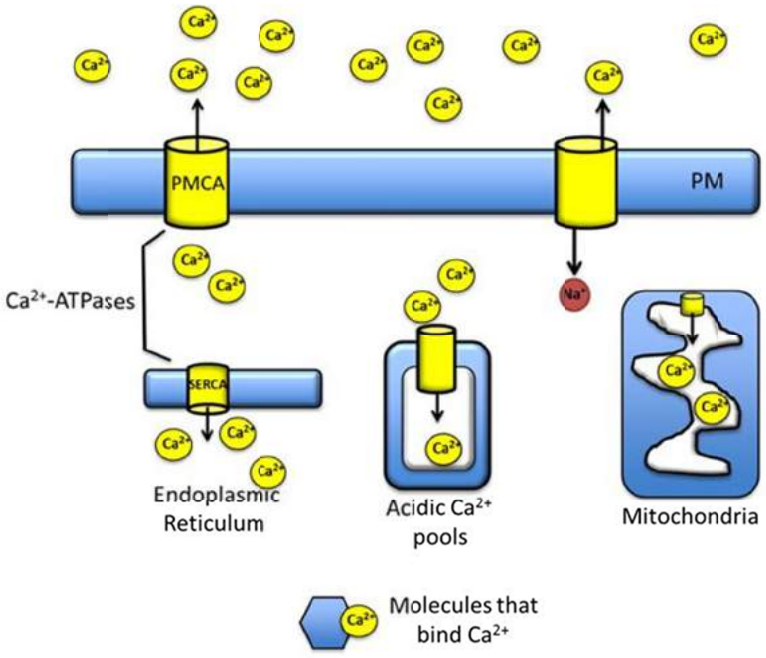

Figure 2: The main mechanisms through which eukaryotic cells regulate and maintain very low concentrations of Ca^{2+} in the cytosol. PM - Plasma Membrane, PMCA - plasma membrane Ca^{2+} ATPase, SERCA - sarco/endoplasmic reticulum Ca^{2+}-ATPase.

4.3. MECHANISMS UTILIZED BY THE EUKARYOTIC CELLS TO PROMOTE INCREASE IN FREE INTRACELLULAR CA^{2+}

All animal cells [10], vegetal cells [11], fungi [12] and protozoa [13, 14] have signaling mechanisms modulated by extracellular stimuli which act through proteins on the plasma membrane (receptors) thus leading to an increase in intracellular Ca^{2+} levels through various different ways. There are three main mechanisms utilized by the cells to promote intracellular Ca^{2+} increase in response to extracellular stimuli: Voltage-dependent Ca^{2+} channels (VDCC) or voltage-gated Ca^{2+} channels (VGCC), ligand-gated Ca^{2+} channels and intracellular Ca^{2+} channels (Ryanodine receptor and $InsP_3$ gated Ca^{2+} channels).

4.3.1. Voltage Gated Ca^{2+} Channels

The membrane potential is a result of a difference in the electrical charge (electrical potential) between the two sides of a membrane. This potential occurs due to an excess of positive ions on one side of the membrane and an excess of negative ions on the other side. The membrane potential is in the range of -40 mV to -80mV (for neurons) and this potential is maintained through the activity of the Na^+/K^+-ATPase pumps (proteins which transport sodium and potassium across the plasma membrane using ATP as an energy source). This transport creates two concentration gradients: one gradient for sodium, which is found in higher extracellular concentrations as well as a gradient for potassium, which has much higher intracellular concentrations. In addition, there is a specific transmembrane potassium channel, which allows the specific diffusion of K^+ through the membrane down the concentration gradient promoted by the Na^+/K^+-ATPase (Fig. **3**). When K^+ leaks through the channel, one positive charge moves out of the cell thus leaving negative charge inside the cell and adding one positive charge to outside. However, this mechanism alone is not enough to maintain the membrane potential with positive charges outside and negative charges inside. The Na^+/K^+-ATPase plays an essential role in regulating the resting membrane potential by pumping 3 sodium ions out of the cell (adding 3 positive charges outside and leaving 3 negative charges inside) and 2 K^+ ions into the cell (adding 2 positive charges inside and leaving 2 negative charges outside). The movement of these two ions across the plasma membrane leads to an overall negative charge inside and positive charge outside the cell.

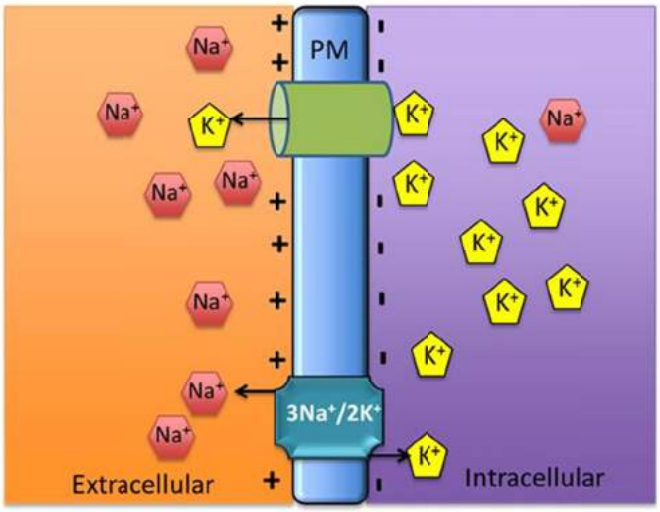

Figure 3: Differences in the concentration of ions on the intracellular and extracellular sides of the plasma membrane generate a membrane potential. This membrane potential is mainly generated by the concentration gradients of two ions: the concentration gradient of potassium (K^+) ions decreases from inside to outside the cell while concentration of sodium (Na^+) ions is greater on the extracellular side of the plasma membrane. Chloride (Cl^-) ions are also present in higher extracellular concentrations. Although these are not discussed in this chapter, they are also involved in generating the resting membrane potential of the cell.

All eukaryotic cells maintain a resting membrane potential and, specifically in excitable cells such as muscle cells and neurons, the membrane potential is used for signaling between different portions of the cell. The signaling occurs by activation or inhibition of specific ion channels on the plasma membrane, which generate local changes in the potential and evoke ion currents (electric current). This allows a very fast flow of ions from one side of the plasma membrane to the other thereby promoting a quick disruption of membrane polarity. There are several different types of Ca^{2+} channels on the plasma membrane, known as voltage-dependent Ca^{2+} channels (VDCC), which are sensitive to this depolarization and are activated under these circumstances (Fig. **4**). Voltage-dependent Ca^{2+} channels are extremely important for fast signal transduction in both excitable and non-excitable cells. The VDCCs are also slightly permeable to Na^+ ions; however, this permeability is 1000-fold smaller than the permeability to Ca^{2+} ions. The modulation of VDCCs is involved in neuronal excitation, gene expression, enzyme functionality and neurotransmitter release, among others. These channels are formed by up to 5 subunits (α_1, $\alpha_2\delta^1$, $\beta_{1\text{-}4}$ and γ) and respond to

membrane depolarization by opening and allowing Ca^{2+} influx through the plasma membrane. VDCC are divided into 3 different families based on their similarities: Ca_V1, Ca_V2 and Ca_V3. The Ca_v1 family corresponds to a class of receptors sensitive to dihydropyridine (**L**-type channels), the Ca_v2 family corresponds to receptors sensitive to spider and/or cone snail toxins (**N**, **P/Q** and **R** types) and the Ca_v3 family represents a group of calcium channels which have low voltage and transient activation (**T -** type) (Table **1**). Interestingly, several different approaches including knockout mouse models, human mutations and pharmacological drug treatments suggest a fundamental role for VDCCs in several neurodegenerative diseases which will be discussed in more details later in this chapter.

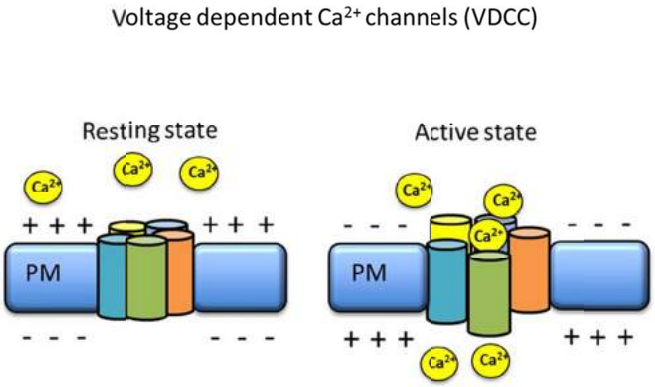

Figure 4: Voltage-gated Ca^{2+} channels showing the "resting" state (inactive) and the active state. PM - plasma membrane.

Table 1: Classification of VDCC

Family	Type	Most found in	Blockers
Ca_v1	L (Long-Lasting)	Skeletal, retina, cardiac cells, dendrites of cortical neurons	Benzodiazepine (*e.g.,* Diltiazem) Dihydropyridine (*e.g.,* Nifedipine) Phenylalkylamines (*e.g.,* Verapamil
Ca_v2	N (Neural)	Brain and peripheral nervous system	Conotoxin
Ca_v2	P/Q (Purkinje)	Purkinje neurons in the cerebellum and cerebellar granule cells	Agarotoxin
Ca_v2	R (Residual)	Neurons and cerebellar granule cells	SNX 482
Ca_v3	T (Transient)	Neurons, bone	Ethosuximide

4.3.2. Ligand-Gated Ca^{2+} Channels

Ligand-dependent channels (LCGD) or ligand-gated ion channels (LGICs) are one class of proteins that have binding sites for specific ligands. They are found on the plasma membrane and are composed of 4 or 5 subunits in various combinations depending on the receptor. These channels can be opened or closed in response to several groups of ligands including proteins, amino acids and hormones. These channels modulate ion flow across the plasma membrane and are thus considered ionotropic receptors. The ion movement through the channel is regulated by ligand binding and is usually very selective to one, or sometimes more, ions including K^+, Cl^-, Na^+ and Ca^{2+} (Fig. **5**).

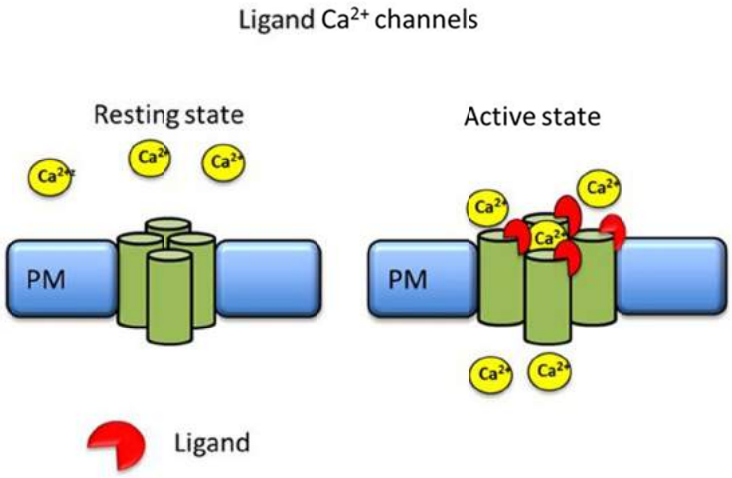

Figure 5: Ligand-dependent Ca^{2+} channels are modulated by various different ligands including hormones, peptides and neurotransmitters. PM - plasma membrane.

There are three different families of ligand-gated channels, which are divided based on their structure and subunit composition:

- **Cys-loop receptors** - these receptors are usually pentameric and characterized by a loop formed by a disulfide bond and two cysteine residues. Included in this family are the gamma-aminobutyric acid receptors (GABA), glycine receptors, serotonin receptors (5-HT), nicotinic acetylcholine receptors (nAchR), and zinc-activated ion channels (ZAC) [15].

- **ATP (Adenosine-5'-triphophate) receptors** - these receptors form a large family of receptors known as purinergic receptors, which open in response to the binding of extracellular adenosine 5'-trisphosphate (ATP). ATP binding to these receptors induces a conformational change in the structure of the protein resulting in the opening of the ion channel and allowing the influx of cations such as Na^{2+} and Ca^{2+} [16].

- **Ionotropic glutamate receptors** - Binding of the neurotransmitter glutamate, modulates the activity of these ion channels. There are three different classes of ionotropic glutamate receptors - NMDA receptor (N-Methyl-D-aspartic acid or N-Methyl-D-aspartate), AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and kainate receptor. They are believed to play crucial roles in neuronal plasticity, learning and memory; however, dysregulation of their function can be neurotoxic and appears to be linked neurodegeneration [17].

4.3.3. $InsP_3$-Gated Ca^{2+} Channels (Inositol Trisphosphate Receptor)

Unlike the other Ca^{2+} channels discussed above (Voltage-gated and ligand-gated calcium channels), the $InsP_3$-gated Ca^{2+} channel or $InsP_3$ receptor ($InsP_3R$) is present inside the cell specifically at the membrane of the endoplasmic reticulum. The $InsP_3R$ has an $InsP_3$ binding domain and binding of $InsP_3$ to the receptor evokes a conformational change in the receptor. This leads to Ca^{2+} efflux from the endoplasmic reticulum to the cytosol as the channel pore opens. $InsP_3$ generation is the most common, if not the only, mechanism through which events at the plasma membrane lead to a mobilization of Ca^{2+} from intracellular stores. The role of $InsP_3$ as a second messenger was first reported in 1983 [18] and, since then, several studies have been done to understand the mechanisms regulating receptor synthesis and degradation as well as Ca^{2+} mobilization from the endoplasmic reticulum [19].

$InsP_3$ formation occurs through a cascade of reactions triggered by the activation of a plasma membrane receptor by an agonist (*i.e.* chemical that binds to a receptor and evokes a response by the cells - *e.g.* hormones, growth factors,

neurotransmitters and drugs). The receptors responsible for $InsP_3$ formation are known as G protein-coupled receptors (GPCRs), serpentin receptors or G protein-linked receptors. G protein-coupled receptors are found only in eukaryotes and many ligands can promote the activation of these receptors including odors, pheromones, hormones, neurotransmitters, peptides and even light.

These receptors form the largest family of cell surface receptors and are present in all eukaryotes. Approximately 4-5% of all proteins coded by the human genome are GPCRs and over 90% of GPCRs are expressed in the brain [20]. GPCRs are involved in several diseases and 40% of all drugs on the market are designed to regulate their function [21].

The interaction between the GPCR and the agonist activates another class of proteins known as G proteins, which are composed of 3 different subunits (α, β and γ). In its inactive form (not bound to the agonist), the G protein associated with the receptor is reversibly bound to guanosine diphosphate (GDP) and the three subunits form a heterotrimer. However, upon GPCR activation, the G-protein exchanges a molecule of GDP for GTP (guanosine triphosphate) at its α-subunit. This promotes the dissociation of the G-protein from the receptor and dissociation of the trimeric G-protein into its two constituent signaling complexes: the α subunit and the $\beta\gamma$ dimer. There are 3 main sub-classes of Gα ($G_{\alpha s}$, $G_{\alpha i/o}$ and $G_{\alpha q/11}$,), which have distinct signaling cascades, second messengers and functions (Table **2**). In terms of Ca^{2+} signaling, $G_{\alpha q/11}$ is involved in the transduction of external signals sensed by the transmembrane receptors. When an agonist activates a Gq-coupled receptor, $G_{\alpha q/11}$ is activated and exchanges GDP for GTP. This process induces the activation of another protein called phospholipase C, which acts to cleave a minor phospholipid component of cell membranes, phosphatidylinositol 4,5-bisphosphate (PIP_2). This leads to the formation of diacylglycerol (DAG) and Inositol 1,4,5-triphosphate ($InsP_3$, IP_3). $InsP_3$ binds to receptors on the endoplasmic reticulum ($InsP_3$ receptors) and induce Ca^{2+} release (Fig. **6**), which can modulate several proteins inside the cell including enzymes, transcription factors and ion channels. Moreover, intracellular Ca^{2+} increase can activate Protein Kinase C (PKC), which can also be activated by DAG, and is involved in many physiological processes such as memory, secretion of neurotransmitters, neuronal excitation, and muscular contraction, among others.

Table 2: Gα protein Sub-class and functions

Sub-Class	Second Messenger Invovled	Agonists	Function
Gαs	Stimulation of Adenylyl cyclase and increase in cAMP (cyclic Adenosine Monophosphate) levels	Dopamine, Acetylcholine, Adrenaline, Melatonin, Serotonin	Increase in heart rate; breakdown of glycogen and fat; activation of enzymes and transcription factors; Cellular proliferation
Gαi/o	Inhibition of Adenylyl cyclase reducing cAMP levels	Acetylcholine, Dopamine, Melatonin, Serotonin, Glutamate, Adrenaline	Decrease in heart rate; modulation of ion channels on the plasma membrane
Gαq/11	Stimulation of Phospholipase C (PLC), increase in $InsP_3$ levels and mobilization of Ca^{2+} from endoplasmic reticulum.	Acetylcholine, Serotonin, Melatonin, ATP, Glutamate	Muscle contraction; cellular proliferation; secretion; learning; anxiety

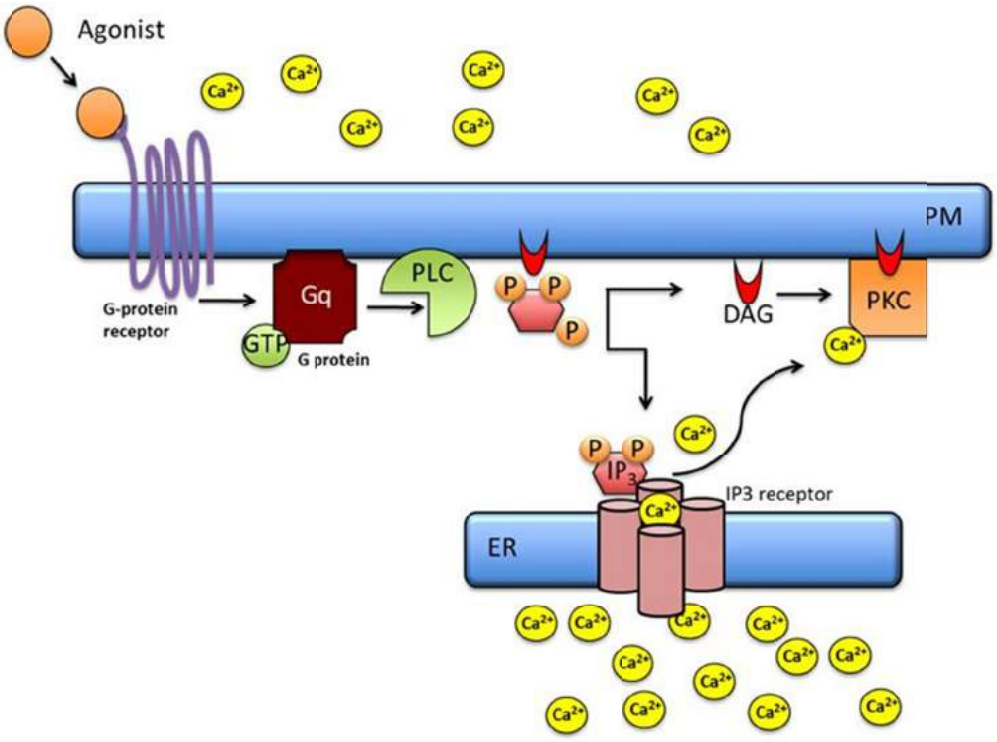

Figure 6: Modulation of G protein-coupled receptors promote an increase in intracellular Ca^{2+} levels by activating of PLC which, in turn, produces $InsP_3$. $InsP_3$ binds to specific receptors in the endoplasmic reticulum leading to mobilization of Ca^{2+} from these intracellular stores.

4.4. CALCIUM AND NEURODEGENATION

In this chapter, we have discussed calcium homeostasis, regulation and signaling. In addition, we discussed the roles of Ca^{2+} ions in several physiological processes. In this part of the chapter we will focus on how the dysregulation of Ca^{2+} homeostasis can promote neuronal death, which can contribute to neurodegenaration.

Neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease (HD) and Multiple Sclerosis (MP) present a huge medical, economic and social problem. According to the Alzheimer's Association, the costs for treatment of people with Alzheimer's disease and other dementias in the USA were around $183 billion in 2011 and the projections suggests that this cost will increase to $1.1 trillion in 2050. Regardless of the tremendous annual investment into treatments and research, the process of neurodegeneration is not well understood and a substantial effort has been put forth in order to develop effective treatments and identify novel targets which may be used for specific therapeutic strategies and drug development to reduce disease severity. Interestingly, despite the pathological and physiological differences between neurodegenerative diseases, there are peculiar similarities indicating that neuronal Ca^{2+} signaling homeostasis is dysregulated in most of these disease states. In neurons, under normal physiological conditions, Ca^{2+} flux across the plasma membrane and mobilization from intracellular stores play fundamental roles in neuronal processes such as neuronal differentiation and plasticity, synaptic transmission, neurotransmitter release, neurite outgrowth, synapse formation and neuronal survival. However, several studies have demonstrated that dysregulation of Ca^{2+} homeostasis promotes neuronal death and could be involved in neurodegeneration [22, 23]. Importantly, the majority of neurons in our brain differentiated at birth and are not regenerated or replaced as we age. Due to the fact that Ca^{2+} homeostasis and signaling can be affected by age, it is logical to speculate that neurons from elderly individuals are more susceptible to Ca^{2+} dysregulation, an idea that would explain the high incidence of neurodegenerative diseases in aged persons. Interestingly, variations in Ca^{2+} homeostasis and signaling were found in studies comparing neurons from young and old rodents. This difference is essentially due

to an increase in Ca^{2+} release from intracellular stores, an increase in Ca^{2+} influx through the Ca^{2+} channels at the plasma membrane [24, 25] and by the deficiency in Ca^{2+} buffering by mitochondria in aged neurons as compared to young neurons. Remarkably, dysregulation of Ca^{2+} buffering, dysregulation of mitochondrial and/or ER Ca^{2+} storage capacity and changes in expression and/or activity of Ca^{2+} channels are associated with most neurodegenerative diseases. Therefore, the mechanisms involved in Ca^{2+} dysregulation are being explored as possible targets for neurodegenerative diseases treatment [23].

4.4.1. Ca^{2+} and Alzheimer's Disease

Biochemically, Alzheimer's disease is characterized by oligomers and amyloid plaques produced by β amyloid peptides (Aβ), and neurofibrillary tangles formed by the hyperphosphorylated form of the protein Tau, in brain cells (See chapter 2). Interestingly, Aβ accumulation has been associated with Ca^{2+} dysregulation and activation of Ca^{2+}-dependent signaling pathways leading to cell death. For example, patients who suffer from sporadic Alzheimer's disease present greater activation of Ca^{2+}-dependent enzymes, such as proteases from the calpain family. When activated by intracellular Ca^{2+}, calpains cleave several proteins involved in maintaining the normal physiology of neurons thus resulting in neuronal death (apoptosis) [26]. Aβ can also induce Ca^{2+} influx into neurons by inducing the formation of a pore in the plasma membrane thus leading to neuronal death [27]. Aβ peptides also alter and/or impair PMCA (plasma membrane Ca^{2+}-ATPase) functionality resulting in depolarization of the membrane and Ca^{2+} toxicity *via* activation of both NMDAR (N-methyl-D-aspartate receptor) and voltage-gated Ca^{2+} channels [28]. In addition, there is an increase in the amount of Ca^{2+} being stored and released from intracellular stores (endoplasmic reticulum) by $InsP_3$ [29] and a decrease of expression of the Ca^{2+} buffer calbinding in AD models [30].

4.4.2. Calcium and Parkinson Disease

Parkinson's disease (PD) is caused mainly by a progressive loss of the dopamine neurons associated with deficiency of the neurotransmitter dopamine in specific parts of the brain - such as the striatum. Interestingly, few studies have demonstrated an association between Ca^{2+} dysregulation and PD. Some studies showed an increase of Ca^{2+} levels in mitochondria [31], excessive Ca^{2+} influx *via*

glutamate receptor and or voltage-gated Ca^{2+} channels and mobilization of Ca^{2+} from intracellular stores. Interestingly some L-type calcium channel blockers have been shown as a potential neuroprotective factor in PD [32].

4.4.3. Calcium and Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease affecting basically motor neurons. The affected motor neurons have signs of organelle fragmentation, damage by free radicals, impairment of axonal protein and disturbance on intracellular Ca^{2+} homeostasis. Some studies have shown an increase in Ca^{2+} concentration in motor nerves terminals in human ALS muscle. In ALS there is an overload of Ca^{2+} in mitochondria, excitotocity mediated by intracellular Ca^{2+} influx *via* AMPA (glutamate receptors) and increase of Ca^{2+} mobilization from endoplasmic reticulum [33].

4.4.4. Calcium and Huntington's Disease

Huntington's disease (HD) is a neurodegenerative disorder characterized by motor changes among others such as cognitive and psychiatric changes. Huntington is a genetic disease in which mutations in specific gene - huntingtin gene - is responsible for the phenotypes. Huntingtin is a protein involved in several physiological processes including Ca^{2+} homeostasis [34]. It was demonstrated an increase of intracellular Ca^{2+} release in response to modulation of glutamate receptors (metabotropic glutamate receptors type I) in HD models. In addition, this dysregulation in the Ca^{2+} levels would be associated to neurodegeneration in HD. Some mechanisms responsible for Ca^{2+} dysregulation have been described: a) mutation in the protein Huntingtin would be involved in the sensitization of NMDA receptors promoting an increase of Ca^{2+} influx [35]; b) mutation in Huntingtin could lead to a sensitization of $InsP_3$ receptors and destabilization of mitochondrial Ca^{2+} regulation [36].

4.4.5. Calcium and Multiple Sclerosis

Multiple sclerosis is a neurodegenerative disease in which the myelin sheath (fatty layer around the axons of the brain and spinal cord which work as an electric insulator) is damaged. This neurodegenerative disease is characterized by inflammation, demyelination and the death of oligodendrocytes, white matter cells

that produce myelin for the myelin sheath [37]. Despite extensive studies regarding molecular aspects of multiple sclerosis, little is known about Ca^{2+} signaling and homeostasis in this pathological condition. However, it has been demonstrated that Ca^{2+} homeostasis is dysregulated as several different channels including PMCA2 (plasma membrane Ca^{2+} ATPase 2), $Na^{2+/}Ca^{2+}$ exchangers and SERCA have been implicated as important players in the progression of this neurodegenerative disease. Moreover, several studies using animal models have suggested that extracellular calcium influx, *via* VGCCs, can contribute to the damage in the white matter. The fact that,calcium channel blockers have been found to have a positive effect ameliorating multiple sclerosis symptoms in mice, lends further support to this idea [38].

CONCLUSION

As described in this chapter, a fine regulation is needed to maintain the Ca^{2+} homeostasis in the cells. The maintenance of the Ca^{2+} homeostasis as well as the controlled intracellular Ca^{2+} increase plays critical roles in fundamental functions of neuronal cells. However, increase the oxidative stress and accumulation/aggregation of proteins related to neurodegenerative disorders such as Alzheimer's, Parkinson, Huntington and prion diseases compromise Ca^{2+} homeostasis system leading to neuronal loss, impairment of neuronal plasticity and neurodegeneration. Despite Ca^{2+} dysregulation is not the always first step in neurodegeneration, most or almost all neurodegenerative diseases have, at some point of their progress, an impairment of intracellular Ca^{2+} system leading to neuronal loss. Due the importance of Ca^{2+} regulation a better understanding of molecular and cellular mechanisms to prevent disturbances in Ca^{2+} homeostasis may open new avenues for therapeutic treatment in neurodegenerative diseases such as Parkinson's, Alzheimer's, ALS and prion diseases.

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CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

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CHAPTER 5

Protein Misfolding and Propagation in Neurodegenerative Diseases

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Abstract: Protein misfolding is the hallmark of a large number of neurodegenerative diseases and is characterized by the presence of amyloid inclusions composed of aggregates of misfolded proteins in specific areas of the brain. The formation of those aggregates involves a multistep process that is exacerbated during periods of cellular stress. Cells have several mechanisms to regulate protein quality control that also serve as a defense line to prevent the accumulation of misfolded proteins; failures in these defenses are frequently involved in neurodegeneration. Another intriguing feature of neurodegenerative diseases, which have misfolded proteins as etiological agents, is the presence of similarities with prion diseases. Prions are unconventional infectious agents composed entirely from a misfolded form of a native protein that has the capacity to provoke and propagate to neighboring cells or even to other organisms. Nowadays, a large body of evidence has shown that most of the misfolded proteins found in degenerated brains behave as prion-like proteins, promoting misfolding and consequently, the aggregation of native protein forms which can spread to other cells or brain regions. However, unlike prion diseases, the prion-like properties of misfolded proteins are unable to naturally infect other organisms. Taken together, neurodegenerative diseases share many characteristics, of which protein misfolding is the most important. This feature has huge therapeutic implications since it raises the possibility to treat different diseases with drugs targeted to impair, block or revert protein misfolding.

Keywords: Protein aggregates, oligomers, fibrils, amyloid plaques, tangles, Lewy bodies, chaperones, protein degradation, oxidative damage.

5.1. INTRODUCTION

The large majority of neurodegenerative diseases addressed in this book share a

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common mechanism: the misfolding of certain proteins which usually lead to their improper clustering, causing cellular toxicity and frequently promoting neuronal death. The type of protein misfolding and the affected brain region will determine the type of neurodegeneration. For example, a hallmark of Parkinson's disease (PD) is the presence of Lewy's Bodies that are composed by aggregates of α-synuclein in the cytoplasm of neurons from the Substantia nigra.

The aim of this chapter is to present the most accepted hypothesis about the mechanisms governing protein misfolding (PM) which has been a matter of intensive studies in the last decades. One intriguing aspect of PM is the capacity that one misfolded protein molecule has to provoke or induce the misfolding of its native counterpart. This phenomenon was first described in prions, infectious pathogens composed exclusively of proteins with the ability to convert the normal protein isoform found in many cells (but especially in neurons) into *de novo* prions. In recent years, this wave of aggregate spreading was observed in other neurodegenerative diseases, such as Parkinson and Alzheimer's diseases where α-synuclein and tau behave as prion-like particules converting their native proteins into the misfolded form. Until now, there is no evidence showing that these diseases, such as prion diseases, are infectious, but there is no denial that other misfolded proteins play a role in nucleating aggregates, which in turn can catalyze the onset of diseases [1].

5.2. PROTEIN MISFOLDING

The function of a given protein is dependent on its structure, which in turn is dependent on its proper folding. At this point, two important considerations can be drawn from the misfolding process: it can generate loss- and/or gain-of-function. Loss-of-function occurs when misfolded proteins become unable to perform their function and, consequently, resulting in an irreversible cell dysfunction status that can lead to cell death. Conversely, gain-of-function phenotype is related to an acquired toxic activity of aggregates themselves.

The anatomopathological evidence of PM in neurodegenerative diseases is the presence of amyloid structures deposited in the extracellular space or in the cytoplasm. The formation of these structures involves a multistep process that is

not completely understood. The evidence suggests that the initial event is the formation of oligomers, molecular complexes composed of a few monomers of a misfolded protein. The formation of protofibrils occurs by direct interaction among several oligomers, which are the precursor of fibrils, the main constituents of amyloid structures. Fibrils are stable entities which act like seeds, serving as active nucleus accelerating polymerization and promoting amyloid growth. The driving forces involved in fibrillar aggregation are the hydrophobic or polar hydrogen interactions among side-chain groups [2].

The major misfolded proteins involved in the most prevalent neurodegenerative diseases include tau, β-amyloid and α-synuclein. Tau is the most commonly misfolded protein found in neurodegenerative diseases, including Alzheimer's (AD), in some cases of prion diseases (Gerstmann–Sträussler–Scheinker syndrome or GSS) and Parkinson's, Frontotemporal dementia (FTD) and Pick's disease, among others. Tau is a protein that is involved in the stabilization of microtubules, controlling cytoskeleton dynamics and its function is modulated by phosphorylation. In all cases of brain neurodegeneration, tau is found in its misfolded form due to extensive phosphorylation, which causes its aggregation and accumulation in filamentous structures called tangles, which are essential for neurodegeneration [3].

β-amyloid is a small peptide derived from the amyloid precursor protein (APP) through the sequential activity of proteases named secretases. While the physiological function of Aβ remains elusive, much evidence has addressed that Aβ oligomers are the most important toxic agents in AD and major components of amyloid plaques, a hallmark of this disease [3].

α-Synuclein is the most important component found in the neuronal inclusions termed Lewy's bodies, the major neuropathological feature of Parkinson's disease and related disorders. The normal protein is mainly found in synaptic regions and plays a role in synaptic vesicles cycle in active zones [4].

Several cellular processes control protein folding and can be directly involved in the pathogenesis of neurodegenerative diseases, among which are mechanisms associated with transcription and protein synthesis, post-translational modifications and degradation, besides chaperones.

5.2.1. The Molecular Chaperones

Chaperones are highly conserved proteins specialized in assisting the folding or assembly of other proteins, but are not part of the final functional structure. Since chaperones are able to target misfolded proteins, they act like neuroprotective agents, preventing initial aberrant protein interactions that culminate in aggregation, which trigger pathogenic cascades [5]. Contained within their amino acid sequence, proteins have, *A priori,* all the information necessary to order their three-dimensional structure. However, the *in vivo* microenvironment (intracellular or extracellular space) is filled by several molecules that can contribute to spontaneous misfolding and aggregation. Thus, very primitive organisms, such as bacteria, evolved the chaperone systems to prevent improper protein interactions that can trigger incorrect folding. These improper interactions are especially abundant in stress conditions, thus chaperones are particularly necessary in protection against stress.

One of the most studied areas of cellular stress is temperature elevation, in such a condition, a cellular program called *heat shock response* is activated, increasing the synthesis of heat shock proteins (HSP), a subset of chaperones essential for recovery from cellular stress. One important implication of temperature maintenance in a disease affected brain is that during fever episodes, for example, there may be a change in balance to favor misfolding, thus aggravating the clinical situation of a patient. Activation of HSP is not limited to temperature elevation; other environmental stress agents, such as chemicals toxins, ultraviolet light exposure, starvation, oxygen or water deprivation, promote their synthesis and activity to buffer protein misfolding. Unfortunately, under certain pathological conditions, the capacity to control protein folding is surpassed and misfolded proteins start to accumulate. The involvement of chaperones in neurodegeneration is supported by the presence of these proteins in many amyloid inclusions from degenerated brain [5].

5.2.2. The Protein Degradation Machinery

Besides controlling protein folding, cells have evolved other mechanisms to destroy misfolded proteins: a degradation machinery commonly active in all cell types, the ubiquitin-proteasome system (UPS) and lysosome mediated autophagy. Every

protein has a period of existence determined by several conjunctures and the ratio of synthesis and degradation is referred to as protein turnover, varying from minutes to several days, depending on the protein. When chaperone capacity is exceeded, protein degradation machinery acts as a second trench to clean up misfolded proteins. The major cellular pathway involved in protein degradation is the UPS, which is characterized by several enzymes that recognize proteins to be degraded in cytoplasm by the conjugation of a small protein called ubiquitin. Ubiquitinated proteins are delivered to a complex called proteasome that is composed of a set of proteases (enzymes specialized in the degradation of other proteins), which cleaves protein substrates to small peptides that can be recycled by cells [6].

Proteins can also be degraded in lysosomes, which are small sphere-like organelles with a highly acidic lumen and several digestive enzymes (proteases, glycosidades, nucleases, lipases, among others). Proteins that are digested by lysosomes have to be associated to intracellular vesicles that fuse to lysosomes releasing their content into the organelle. Extracellular and membrane-bound misfolded proteins can be cleared by this system [7].

Together with chaperones, protein degradation machinery is a part of the quality control mechanism specialized in recognizing and repairing (chaperones) or eliminating misfolded proteins (the ubiquitin-proteasome system and lysosomes). Thus, cells maintain protein quality control in a robust and redundant fashion, sustaining a complex system of multiple-type components to prevent any potentially toxic misfolding event.

The amyloid plaques found in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, for example, are enriched in chaperones and components of the degradation system. The presence of these proteins reflects at least two possibilities that can occur simultaneously: the first is a failed attempt of cells to revert misfolding or clear the aggregates, and second, the interaction among misfolded proteins can be irreversible. An important therapeutic implication arises from both possibilities, since cells are very sensitive to the presence of these aggregates and/or their intermediates. Can we disturb cell death pathways in order to permit cells to tolerate the misfolded proteins? Some studies demonstrate that stress pathways are activated by the presence of misfolded

proteins, but the pharmacological inhibition of such pathways can prevent cell death [8,9].

5.3. CELLULAR INSULTS COMMONLY ASSOCIATED WITH PROTEIN MISFOLDING

One of the most accepted hypothesis about the origin of protein misfolding and aggregation is the involvement of the excessive generation of free radicals, such as reactive species of oxygen (ROS) and nitrogen. These radicals are formed as a by-product of oxidative metabolism, having mitochondria as a pivotal agent.

5.3.1. Mitochondrial Dysfunction

Mitochondrial dysfunction has been associated with pathogenic processes related to neurodegenerative diseases. Mitochondria have a major role in cellular function through extensive production of ATP by oxidative phosphorylation. Besides its role as cell dynamos, mitochondria are also responsible for other cellular functions, such as calcium ion storage, lipid metabolism and regulation of programmed cell death [10]. A major implication of this deep integration with cell physiology is that in many cases mitochondrial dysfunction results in cell death. Aging is one important cause of decreased mitochondrial function. Neurons have evolved specialized mechanisms to prevent mitochondria dysfunction and, consequently, to decrease the risk of excessive oxidative stress. In aging, deficits of antioxidants synthesis occur favoring the occurrence of oxidative insults [10].

A dramatic consequence of mitochondrial dysfunction is the formation of ROS, since this organelle consumes large amounts of oxygen during oxidative phosphorylation. Electrons can escape from the electron transport chain leading to the formation of superoxide anions (O^{2-}) that are very reactive and generate hydrogen peroxide or hydroxyl radicals. These radicals are able to damage other macromolecules such as protein, lipids and nucleic acids. The generation of misfolded proteins, as a result of extensive oxidative insult, can result in cell death by apoptosis or necrosis [11]. Cells also develop a robust mechanism to self-protect from oxidative insults, such as scavenger molecules and enzymes, which are highly expressed to rapidly quench reactive oxygen species [10].

The most interesting case of oxidative damage associated with protein misfolding is a form of ALS (Chapter 10) linked to a mutation in superoxide dismutase 1 (mSOD1), a pivotal protective enzyme that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. Due to the mutation, the enzyme acquires a conformational instability that causes misfolding and leads to decreased protein function. Moreover, some evidence suggests that even wild-type SOD1 can lose its enzymatic activity under cellular stress, which can be associated with sporadic forms of ALS [12]. A remarkable consequence of the loss-of-function of SOD1 is the increased generation of ROS, which causes motor neuron death associated with ALS degeneration. Interestingly, proteome studies revealed that the levels of at least 50 mitochondrial proteins were altered, indicating a widespread effect of mitochondrial dysfunction in this disease [10].

Another important oxidative event associated with protein misfolding and degeneration involves the metabolism of the neurotransmitter dopamine [13]. Dopamine has a unique chemical structure with a potential risk to be oxidized, even in physiological conditions, which yields the generation of toxic semiquinone radicals from the dopamine catechol group [14]. This oxidative by-product will react with α-synuclein on the surface of synaptic vesicles leading to its oxidization and consequent accumulation [13].

5.3.2. Calcium-Induced Protein Misfolding

Another proposed mechanism that leads to protein misfolding involves the activation of the NMDA receptor, one of the most important receptors to control neuronal function. The NMDA receptor is activated by its ligand glutamate, the major excitatory neurotransmitter of the mammalian brain. When glutamate accumulates in extracellular space, there is an overstimulation of NMDA receptors, which is associated with a pathological condition named excitotoxicity [15]. The NMDA receptor is an ionic channel coupled receptor (also called ionotropic receptor) that is permeable to calcium ions and is required for normal neurotransmission. However, a number of chronic or acute pathological conditions can lead to the accumulation of glutamate in the synaptic cleft, overstimulating the NMDA receptors and causing an overload of cytoplasmic calcium. As discussed in Chapter 4, calcium is a very important second messenger

in cell signaling and its cytoplasmic levels should be kept in low concentrations (pumped to the extracellular milieu or stored in intracellular organelles, such as endoplasmic reticulum and mitochondria). During periods of excessive activation of the NMDA receptor, calcium levels tend to increase, leading to production of damaging free radicals, which in turn will be associated with a cellular stress that usually deregulates quality control processes, increasing the probability of misfolding-prone proteins to progressively accumulate [16].

Another route of calcium influx to cytoplasm is through a transmembrane pore-like formation which is self-assembled by misfolded proteins that allows a deregulated calcium entry that promotes all the aforementioned toxic events [17]. These pore-like structures were described with mutant forms of prion protein as well as β-amyloid [18] and α-synuclein [19].

5.3.3. Inflammation

Another source of cellular stress in neurodegenerative diseases is the participation of the immune system. The misfolded protein aggregates act as irritants, eliciting a chronic and intense inflammatory reaction that can lead to neuronal death [2]. The presence of extensive areas of astrocyte proliferation and microglial activation around inclusions is the main evidence of chronic inflammatory reaction. Moreover, accumulation of inflammatory protein in cerebral amyloid inclusions was also observed, including complement proteins, inflammatory cytokines, proteases and protease inhibitors. Attempts to treat neurodegeneration were made using nonsteroidal anti-inflammatory drugs (NSAIDs) in animals and humans [2, 20]. Clinical trials were held for short-term periods (maximum of two years) with controversial outcomes. Most of them presented minimal effects, with benefits only in patients in very early stages of the AD process. Long-term clinical trials are necessary to evaluate if NSAIDs would be safe and beneficial to AD patients [21].

5.3.4. Mutation and Gene Amplification

Beyond the factors described above, other mechanisms can also contribute to protein misfolding. Mutations are perhaps the most studied causes of protein misfolding, since changes in the protein sequence usually present dramatic

consequences in protein conformation. There are numerous examples: APP, tau, α-synuclein, Prion protein, SOD and Huntingtin among other less famous proteins. Moreover, another genetic component that may contribute to neurodegeneration is gene amplification, which is usually associated with protein overexpression. At least two important examples illustrate the importance of gene amplification. The best characterized case is the increased incidence of Alzheimer's disease in Down syndrome individuals who have an extra copy of the APP locus present on chromosome 21. Down syndrome patients have increased rates of APP synthesis, which favors the probability of β-amyloid formation [22]. Another important example of gene amplification associated with neurodegenerative disease was verified in a family who has a α-synuclein locus triplication, which was associated with Parkinson disease [23].

5.4. SELF-PROPAGATION OF MISFOLDED PATHOGENIC PROTEINS

In the last few years, another common aspect from neurodenerative diseases has been discussed, a peculiar characteristic which was first described in transmissible spongiform encephalopathies, or prion diseases. Prions (PrP^{SC}) are unconventional infectious agents composed entirely from the misfolded form of a normal and ubiquitous protein called cellular prion protein (PrP^{C}). Interestingly, PrP^{C} can be directly converted by PrP^{SC} (in a mechanism still poorly understood) into the misfolded form, promoting the formation of new prions. Prions can infect other individuals and even other species, causing a fatal neurodegeneration. For the discovery of the prion paradigm, Nobel Prizes were awarded on two separate occasions, first to Carleton Gajdusek in 1976 for describing kuru, an infectious brain disease of the Fore people from Papua, New Guinea and then later to Stanley Prusiner in 1998 for elucidating the nature of prions. For many years, the prion paradigm of information that can be transmitted without nucleic acid was a matter of debate in the scientific community, but recently the unique feature of prions have begun to be considered in other neurodegenerative diseases [24].

As discussed before, protein misfolding and aggregation in other neurodegenerative diseases follow the prototypic prion disease, involving a pattern of seeding-nucleation with the consequent formation of small aggregates that culminate in oligomer assembly and fibrils as the final product, the main

components of amyloid plaques [24]. Additionally, *in vitro* evidence has shown that several proteins implicated in human diseases have their aggregation accelerated when in contact with experimental seeds, suggesting that aggregation-prone proteins have the intrinsic ability to be transmissible. These findings are also supported by *in vivo* models such as cell cultures and transgenic mice, showing that transmissibility of protein misfolding has a prion-like behavior. We cannot discard that sporadic cases of these diseases can be explained by this model; however, this has not been experimentally demonstrated [25].

Another common feature among most neurodegenerative diseases is its spread in a progressive manner, starting in a small brain region and reaching distant areas of the brain, supporting the idea that this spread is accompanied by the diffusion of a putative pathogen. This behavior is described in AD, PD, FTD and Huntington´s disease. This hypothesis was further reinforced by the evidence that PD patients who received fetal mesencephalic nerve cell transplants in order to replenish dopamine-releasing cells (in an attempted stem cell therapy, see more in Chapter 15) presented synuclein inclusions in graft derived cells [26]. This demonstrates that synuclein aggregates can be transmitted from host affected cells to healthy donor cells, consistent with the idea of the spreading of seeds followed by conversion of α-synuclein to a misfolded state. This evidence has a huge consequence in the development of treatment strategies to neurodegenerative diseases based on stem cell therapy, for instance [27].

Accordingly, soluble β-amyloid aggregates derived from transgenic mice were able to induce amyloid plaque formation in wild-type animals [28]. In this context, it is important to keep in mind that the immune system can also be used by aggregates to navigate beyond the brain tissue reaching peripheral nervous system and bloodstream, disseminating through the entire body. In accordance with these ideas, a study has demonstrated that intra-peritoneal inoculation of brain homogenates from AD patients was able to increase the progression of the disease pathology in animal models [29].

The characterization of key mechanisms involved with the secretion, uptake, conversion and toxicity of misfolded protein will shed light to the unknown etiology of many of these diseases, permitting the characterization the etiology of

sporadic cases, which represent around 90% of cases of all neurodegenerative diseases.

5.4.1. Self-Propagating Misfolded Proteins Involved In Neurodegeneration

5.4.1.1. Tau Protein

Tau inclusions found in the diseased brain appear to have a pivotal role in neurodegeneration and several factors can trigger their formation. Is not clear whether tau aggregates can be self-sustained, but increasing evidence suggests that tau inclusions have a pattern of distribution which correlates with aging, suggesting that the process starts at transentorhinal cortex spreading to the hippocampal formation and the neocortex [3]. An implication of being a transmissible agent is the different grades of *virulence,* which is consistent with the idea of prion strains. In the case of tau, strains can be a result of distinct tau isoforms (at least six in humans) [30].

An important observation, however, is that many studies that had shown the putative transmission of protein aggregates in neurodegenerative diseases had their conclusions drawn by correlation data, without direct experimental support, which is insufficient to conclude that transmission, in fact, occurs. For that reason, many researchers are still skeptical about this possibility. However, during the last few years, many scientists have dedicated their efforts to prove this possibility [3]. The most intriguing findings were when it was demonstrated that brain extracts derived from transgenic mice expressing human tau mutations were able to induce the assembly of tau protein aggregates and those injected mice presented a spread pathology to other brain areas resembling the human disease [31].

5.4.1.2. β-Amyloid

β-Amyloid inclusions are present in the extracellular space, unlike other protein aggregates discussed here. However, the mechanisms of aggregate dissemination are similar. Several studies have already shown that the inoculation of brain extracts from AD patients into the brains of transgenic mice promote aggregation and deposition of β-amyloid in a manner consistent with the existence of different β-amyloid strains [32-34]. Similar outcomes were obtained with nonhuman

primates, which also were inoculated with β-amyloid aggregates from human diseased brain and, like prions, synthetic human β-amyloid aggregates were able to induce lesions in transgenic mice expressing human APP protein. The spreading of aggregates through axonally coupled brain regions are also observed in experimentally induced Alzheimer's disease and culminates with widespread areas, such as neocortical and subcortical regions [25].

5.4.1.3. α-Synuclein

As mentioned before, α-synuclein is the major component of Lewy's bodies, a characteristic of PD and correlated diseases. α-synuclein inclusions can present pathologically different filamentous morphologies, suggesting that different strains can also exist, giving rise to different diseases [3]. Deposits of α-synuclein usually appear in enteric and peripheral nervous systems in the early phases of the disease. This evidence suggested that PD can originate outside the CNS, very much like the most famous form prion diseases, the mad-cow-disease that originates by the ingestion of cattle-derived infected meat. The oxidative damage promoted by pesticide rotenone (which affects mitochondria) is associated with the formation of the pathological form of α-synuclein, suggesting that people exposed to this pesticide can be prone to develop PD. Experimental cell-to-cell transfer of α-synuclein inclusions was also successfully addressed using cell cultures and transgenic mice models [3]. In a behavior that parallels what has been observed for β-amyloid, synaptically connected neurons are able to spread α-synuclein aggregates through retrograde transport, as assessed by inoculation in striatum or cortex, producing different patterns of spread [35].

In summary, many misfolded proteins have been studied in the aspect of their putative transmissibility. The examples listed above show that there is a convergence of studies demonstrating that the pathogenesis of neurodegenerative diseases share similarities with prion diseases. However, until now, the remarkable feature of prions, the infectivity capacity to transfer seeds to other organisms, was not demonstrated under natural conditions. One conclusion to be drawn is that transmission capability does not mean that these diseases are infectious, but taken together those findings are helping researchers to explain

intriguing features of these diseases and will also support novel strategies to develop new diagnostic tools and effective therapies for these diseases [24].

5.5. THERAPEUTICS FOR MISFOLDED PROTEINS DISEASES

Considering that misfolding and aggregation are shared features in the pathogenesis of several neurodegenerative diseases, it is conceivable to expect a common therapy targeted to misfolded proteins. Several approaches have been proposed in the last years and are based on at least four strategies: first, using chemicals to help stabilization of the native protein conformation, thus preventing misfolding; second, desestabilization and reversion of protein aggregates; third, compounds with the capacity of competitively blocking protein interactions in order to prevent the formation of aggregates; fourth, increasing the clearance of misfolded proteins through immunization [2, 28]. All these proposed therapies are still being researched and at the moment no compound is commercially available, albeit some molecules are being tested in humans in clinical trials. However, we have to keep in mind that all strategies should be exhaustively tested since many potential adverse effects could arise. Since the process of misfolding is still poorly understood, the attempts to inhibit one step of misfolding could result in the accumulation of putative toxic intermediates worsening the toxicity [36].

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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CHAPTER 6

Mitochondrial Dysfunction and Free Radicals in Neuronal Death

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Abstract: Mitochondria are key organelles with a critical role as the main source of energy supply. Mitochondrial dysfunction can lead to several disorders, including neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases and the aging process. The mitochondrial respiratory chain is the main source of reactive oxygen species and because it is located in the inner mitochondrial membrane, it is also susceptible to oxidative damage, as well as other biomolecules in the vicinity, such as mitochondrial DNA. Nitric oxide is also found in the mitochondrial matrix and is involved in physiological pathways such as induction of apoptosis and generation of nitrosative stress. In this review we will discuss the complex mechanisms involved in the relationship between mitochondrial dysfunction, oxidative stress and neuronal death.

Keywords: Free radicals, mitochondrial dysfunction, neurodegenerative diseases, neuronal death, nitric oxide, mitochondria, apoptosis, mitochondrial DNA, Alzheimer's disease, Parkinson's disease, motor neurons, aging, mutation, electron transport, oxidative stress, oxidative phosphorylation, Huntington's disease, mitochondrial diseases, superoxide radicals, dementia.

6.1. INTRODUCTION

As the population has become older during the last decades, the incidence of neurodegenerative diseases have increased as well as our knowledge about the complex mechanisms involved in the pathogenesis of neurodegeneration, especially neuronal death. Neurodegenerative diseases result from different mechanisms and multiple effects determine the clinical severity and progression of these diseases. Genetic and environmental factors are involved, but among the pathogenic mechanisms, oxidative stress and mitochondrial dysfunction leading to neuronal impairment or death are considered important [1].

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Mitochondria are key organelles present in almost all cell types and due to their critical role as the main source of energy supply, mitochondrial abnormalities can lead to diseases and specific organ disturbance. Neuronal and muscle cells are among the most susceptible to bioenergetic deficiencies, which is reflected by the implication of mitochondrial dysfunction in several neurodegenerative diseases and in the aging process [2, 3]. Mitochondrial abnormality is found as a primary defect or as a secondary event. To understand how mitochondria are affected in these diseases, we first need to review the basic knowledge about mitochondrial structure, function and genetics.

Mitochondria are cytoplasmic organelles composed by two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The OMM delimitates the organelle from the cytoplasmic content while the IMM is a long length membrane with invaginations called cristae, giving a larger surface area. The space between the OMM and IMM is the inter-membrane space and the internal space formed by the IMM is called the matrix, where most of the physiological pathways occur. The main mitochondrial function is performed by the respiratory chain or electron transport chain, located in the IMM, composed by four multimeric enzyme complexes: Complex I (NADH ubiquinone oxido-reductase), Complex II (succinate ubiquinone oxido-reductase), Complex III (ubiquinone cytochrome *c* oxido-reductase) and Complex IV (cytochrome *c* oxidase); and two electron carriers, coenzyme *q* (or ubiquinone) and cytochrome *c*. Reducing equivalents, produced in the Krebs cycle and beta-oxidation, are passed along the electron transport chain, generating energy that is used to pump protons from the mitochondrial matrix into the inter-membrane space. During this process, an electrochemical proton gradient is created and the protons flow back into the matrix through the F_0 portion of Complex V (ATP synthase), generating ATP [2]. This entire process is denominated oxidative phosphorylation (OXPHOS). Mitochondria also have other critical roles including fatty acid oxidation, Krebs cycle, urea synthesis, regulation of amino acid cycling, neurotransmitter biosynthesis, regulation of cytosolic Ca^{2+} homeostasis, control of cell death and necrosis [3].

As a unique organelle, mitochondrion has its own DNA (mitochondrial DNA, mtDNA), a circular, double-stranded molecule with 16,569 bp, containing 13 structural genes encoding subunits of the respiratory chain, 22 tRNA genes and 2

rRNA genes [4]. Since, mtDNA only is not sufficient to provide all the elements for mitochondrial formation and functioning, so both genomes, mtDNA and nuclear DNA, are needed for proper mitochondrial function.

Mitochondrial dysfunction has been observed in several disorders due to a primary or secondary defect. A primary defect leading to mitochondrial dysfunction, is caused by genetic defects leading to abnormalities of the OXPHOS, known as mitochondrial disorders [5]. These diseases are caused by mutations in mtDNA or nuclear DNA, leading to variable phenotypes, with multisystem involvement and frequent neurological manifestations such as infantile encephalopathy, dystonia, optical neuropathy, epilepsy, parkinsonism, peripheral neuropathy and myopathy. However, these phenotypes do not involve an isolated manifestation, as patients usually have other associated manifestations such as cardiomyopathy, heart block, retinitis, neuro-sensorial deafness, hepatopathy and tubulopathy [5]. The severity of mitochondrial dysfunction and mutation load in these patients is much higher than what is found when mitochondrial dysfunction is a secondary event.

6.2. FREE RADICALS IN MITOCHONDRIA

One of the most important factors associated with the development of neurodegenerative diseases is mitochondrial dysfunction caused by oxidative damage. During normal electron flow in the respiratory chain, there is a leakage of 0.1 to 1% of electrons, making the respiratory chain an important source of free radicals [6, 7]. Because respiratory chain complexes are embedded in the IMM, several potential targets of reactive oxygen species (ROS) are in close vicinity, such as mtDNA and iron-sulfur clusters present in respiratory chain complexes. Although electron leakage occurs normally during electron transport through the respiratory chain, the presence of defense mechanisms, such as antioxidant molecules and scavenger enzymes, are able to avoid oxidative damage. However, when the electron flow is disturbed, an increased leak of electrons is expected, generating superoxide anions ($O_2^{\bullet -}$), which leads to formation of H_2O_2 by superoxide dismutases or spontaneous dismutation. Low to intermediate levels of H_2O_2 are involved in the regulation of redox-sensitive signaling and transcription of several physiological pathways, whereas high levels are involved in oxidative

damage [8]. Oxidative damage is more likely to occur with more reactive radicals, generated through other reactions, which include superoxide anion ($O_2\bullet^-$), H_2O_2, hydroxyl radical (•OH) [9] and reactive nitrogen species (RNS), such as nitric oxide (NO), nitrite and peroxynitrite [10]. Any kind of free radical can promote damage to lipids, mtDNA, proteins, especially those containing transition metals, such as iron and copper, which are present in respiratory chain Complex I and IV, respectively. Nitration of tyrosine residues in mitochondrial proteins, such as cyclophilin D (CyPD) and adenine nucleotide translocator (ANT), can also affect the process of apoptosis [9].

NO is a unique radical because of the negative effects as a reactive radical itself and also how it exerts regulatory roles, such as reversible inhibition of Complex IV (cytochrome *c* oxidase) and control of mitochondrial biogenesis [8, 11]. Several studies reported the mitochondrial generation of NO by a mitochondrial nitric oxide synthase (NOS); however, the existence and identity of this mitochondrial NOS are controversial [6, 7, 12-14].

MtDNA is considered more vulnerable due to the lack of protective histones, but the organization of mtDNA molecules in nucleoids provides some protection [15]. Oxidative damage to mtDNA can promote single or double-strand breaks and mispairing leading to point mutations or deletions. Several point mutations, insertions and deletions have been described, but in very low amounts, up to 1%, which is considered too low to affect cellular mitochondrial function. These mutations are age-related and have been considered as a product of oxidative damage, but until now this hypothesis is still a matter of debate [16].

Respiratory chain complexes I and II are more susceptible to ROS due to the presence of several iron-sulfur clusters. The presence of Fe^{2+} can convert H_2O_2 into hydroxyl radical (•OH) or intermediates, which are more harmful [10]. Another form of protein damage is from NO, which can generate peroxynitrite (ONOO-) and other RNS, leading to interruption of the correct electron flow through the respiratory chain by signaling events promoting inhibition of respiration or nitrosative modifications such as protein nitration [10]. Nitration of tyrosine residues in proteins such as CyPD and ANT can induce apoptosis because they are key components of the mitochondrial permeability transition

pore (PTP) [9, 10]. Proteins can also be inactivated when modified by protein carbonylation [9]. All these modifications affect mitochondrial function and can be related to neuronal damage.

6.3. CELL DEATH

Cell death is a part of the cellular process for normal development in the nervous system. However, cell death can be a response to injury, stress or can be associated to pathological conditions, including neurodegenerative diseases [17]. Cell death can occur by three different processes: necrosis, apoptosis and autophagy [15].

Necrosis is the lytic destruction of individual cells, involving cell swelling and rupture of cellular membranes, which is biochemically and morphologically distinct from apoptosis [17].

Apoptosis is an orderly and compartmental dismantling of single cells or groups of cells into consumable components for nearby cells. This programmed cell death is ATP-driven and often signaled by caspases or other caspase-independent forms of programmed cell death. There is no associated cell lysis or inflammation. Caspases can be activated by two ways, by the mitochondrial pathway or by the death receptor pathway. In the mitochondrial pathway, caspase activation is triggered by the release of cytochrome *c* from mitochondria into the cytosol [18].

Autophagy is a lysosomal degradation of damaged or expendable organelles. Cytoplasmic constituents, including organelles, are sequestered into double-membrane autophagosomes, which subsequently fuse with lysosomes where their contents are degraded [19]. Autophagy is part of the physiological processes involving protein and organelle turnover and can also be found in pathological conditions. When autophagy occurs in mitochondria, the process is denominated mitophagy, which is the recycling or elimination of entire dysfunctional mitochondria. Through the process of mitophagy, the subset of mitochondria producing the most reactive oxygen species are also removed, in order to reduce the oxidative stress [20]. Many cell death stimuli can induce more than one mode of cell death depending on the conditions, such as the severity and duration of the stress, redox levels and mitochondrial integrity [18]. In

order to maintain the energetic supply, there is a continuous removal of non-functional mitochondrial subunits by autophagy, but when extensive mitochondrial damage exists, cell death is triggered [15].

6.4. THE AGING FACTOR

The vast majority of neurodegenerative diseases are age-related, *i.e.*, manifestations occur late in life. So it is reasonable to think that the aging process may be an additional factor that predisposes or modulate these diseases. Since the mitochondrial theory of aging was first mentioned [21, 22], several studies have looked at mitochondrial abnormalities in aged tissues. This theory is based on the fact that the respiratory chain is a major source of electrons that are available to react with molecular oxygen and generate ROS. These might cause oxidative damage to the respiratory chain, leading to abnormal electron flow and increased generation of ROS, creating a "vicious cycle". The main evidence supporting this theory is the impairment of mitochondrial respiratory chain enzyme activities [23, 24], increase in oxidative markers in mtDNA [25] and elevation of proportion of mtDNA mutations [26, 27]. These abnormalities were found in aged tissues and mtDNA mutations accumulate in an exponential manner with age [27]. An argument against the involvement of mtDNA mutations in the aging process is that the levels of these mutations are very low (<1% of total mtDNA), which is not sufficient to lead to mitochondrial impairment. Contrarily, because several mutations (deletions, insertions and point mutations) are present in the same cell [27-29], it is also argued that the decline in enzyme activities could be due to many types of mutations together. Others find that although the mutation load is low, mitochondrial impairment in isolated cells could represent a decrease in the homeostatic reserve of aged neurons, explaining their increased vulnerability [30]. Even though other studies found normal mitochondrial enzyme activities in aged tissues [31, 32], *in situ* studies demonstrate that isolated cells do have mitochondrial deficiency, which could be explained by the very small percentage of cells presenting deficiency in the whole tissue [33]. When looking at individual neurons in *substantia nigra,* mtDNA deletions are found in higher levels in aged individuals, although they account for less than 1% [34]. The study of the *substantia nigra* of an 80 year-old individual showed the presence of 30% neurons with cytochrome *c* oxidase deficiency and with more than 60% of mtDNA

deletion [34]. Studies using the *mutator* mouse, an animal model for premature aging with a defective mitochondrial polymerase gamma [35, 36], have demonstrated accumulation of mtDNA mutations and mitochondrial deficiency.

The main hypothesis to explain the accumulation of mtDNA mutations during life is that they appear as a result of oxidative damage. However, this hypothesis is questioned by several studies and until now is not confirmed. For this reason, a new hypothesis has emerged and is based on the idea that mutated mtDNA molecules are already present early in life, originated from primordial cells, and that the increase of mutated mtDNA molecules occurs due to replication of these molecules in post-mitotic tissues [16, 37].

6.5. OXIDATIVE DAMAGE AND MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are characterized by cumulative neuronal damage in specific brain areas that lead to neurological deficits when neuronal loss reaches a critical limit [38]. The most frequent diseases are Parkinson's disease (PD) and Alzheimer's disease (AD), which are associated with respiratory chain deficiencies in Complex I and IV, respectively [39-41]. However, the exact roles of mitochondria in these diseases are yet to be clarified, though great advances have been acquired, especially with studies on animal models. Most recently, abnormalities on mitochondrial morphology and dynamics have been discovered as important factors in the development of neurodegenerative diseases, including Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) [42, 43].

AD is the most common form of dementia and is characterized by the extracellular accumulation of senile plaques and the intracellular deposition of neurofibrillary tangles of hyperphosphorylated tau protein [44]. It is suggested that the amyloid β peptide (Aβ), present in senile plaques, induces oxidative damage with association of complex IV deficiency. Studies on transgenic mice expressing human mutant amyloid precursor protein (APP) found the association of Aβ with mitochondria and mitochondrial dysfunction (lower levels of oxygen consumption and reduced activity of respiratory complex III and IV) [45]. The presence of Aβ within mitochondria in murine model and AD patients provides a

link between mitochondrial dysfunction and pathogenic Aβ [46]. Aβ peptide is a product of APP, which is synthesized in the cell bodies of neurons and is anterogradely transported *via* axons to nerve terminals in the brain [46]. Both APP and Aβ can affect regular mitochondrial function through direct physical interactions with mitochondrial proteins. Studies on transgenic mice demonstrated that APP clogged mitochondrial import machinery causing mitochondrial dysfunction and energy metabolism impairment [47]. The interaction of Aβ with Aβ binding alcohol dehydrogenase (ABAD), a mitochondrial matrix protein, led to increased free radical generation and impaired memory in APP mutant mice [48]. It was also demonstrated that Aβ interacts with mitochondria leading to cytochrome c oxidase inhibition and impaired memory in animal models [49, 50].

Additionally, brains of patients with AD had lower level of presequence protease (PreP), localized in the mitochondrial matrix and characterized as a mitochondrial Aβ degrading enzyme [51]. The reduction was confined to the temporal lobe while other areas are not affected. AD brains also presented reduction of Complex IV activity and higher levels of 4-hydroxynonenal, an oxidative product, suggesting that enhanced ROS production decreased PreP proteolytic activity, contributing to Aβ accumulation in mitochondria, leading to mitochondrial toxicity and neuronal death [51]. It is hypothesized that the accumulation of Aβ leads to reduced mitochondrial membrane potential, including reduced Complex IV activity, both reducing ATP levels, followed by enhanced ROS production. When the inhibition of mitochondrial function has reached a phenotypic threshold and severe energy deprivation appears, the process culminates with mitochondrial and synaptic dysfunction [52].

Mitochondrial dysfunction in PD is more direct. Mitochondrial involvement in PD has long been observed from toxic agents such as MPTP (1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine) that produced Parkinson's-like syndrome. Its metabolite MPP+ enters neuronal mitochondria and selectively inhibits Complex I. Complex I deficiency was also found in platelets, muscle and brain from PD patients [41]. Inhibition of Complex I creates a biochemical environment with increased generation of superoxide, which promotes lipid peroxidation and peroxynitrite mediated protein nitration and nitrosylation, culminating with neuronal apoptosis [53]. Complex I deficiency also causes alpha-synuclein aggregation and

accumulation in dopaminergic neurons, contributing to neuronal death [54]. Furthermore, increased NO levels in mitochondrial matrix, due to activation of mitochondrial NOS or NO diffusion from cytosolic, leads to peroxynitrite mediated nitration, inhibition of Complex I activity, increased production of superoxide and trigger of apoptotic signaling [38]. However, it is still unclear why mitochondrial dysfunction is restricted to dopaminergic neurons [44].

One of the most devastating neurodegenerative diseases is ALS, characterized by progressive generalized muscle weakness, atrophy, swallowing disability and death due to respiratory failure [55]. ALS is caused by degeneration of upper motor neurons in cerebral cortex and lower motor neurons in the brainstem and spinal cord [55, 56]. Most of the patients have the sporadic form (90%), while only 10% are familial cases [56]. Twenty percent of familial cases are caused by mutations in Cu/Zn-superoxide dismutase gene (*SOD1*), as SOD1 is a scavenger enzyme that removes superoxide anions, increased oxidative stress is expected in this form. However, the mechanisms of the toxic effects of mutant SOD1 are not straightforward and still need to be better clarified. In normal tissues, SOD1 is a cytoplasmic enzyme, though mutant SOD1 localizes in mitochondria only in affected tissues [57, 58]. It was demonstrated that the localization of mutant SOD1 in mitochondria is critical for the pathogenesis of familial ALS because it triggers the release of mitochondrial cytochrome *c* followed by activation of caspase cascade, inducing neuronal cell death [57]. Transgenic mice over expressing mutant SOD1 in the mitochondrial intermembrane space show many but not all ALS-like features [59], which means that other factors are involved in the full development of the disease. The proposed involvement of mitochondrial dysfunction and neuronal death can be summarized in: (a) mutant SOD1 accumulates and aggregates in the outer mitochondrial membrane and clogs the protein importation machinery, resulting in mitochondrial dysfunction; (b) aberrant ROS production is induced by mutant SOD1, leading to oxidative damage and impaired respiration and ATP synthesis; (c) mutant SOD1 inhibits apoptosis because binds to and aggregates with anti-apoptotic proteins (cytosolic heat-shock proteins and mitochondrial Bcl-2) [56]. Furthermore, recent studies have demonstrated abnormalities in axonal transport and movement of mitochondria along the axons, resulting in depletion of mitochondria from the

axons and an accumulation of organelles in clusters along neurites [60, 61]. Impaired mitochondrial transport was associated with inability to maintain viable neurites and may be critical in motor neurons, where cellular components have to move long distances throughout axons, such as in ALS [61].

Abnormalities in mitochondrial dynamics and axonal transport have also been reported in HD. This is another example of a severe and devastating neurodegenerative disease, with late onset and progressive course. It is clinically characterized by chorea, psychiatric disturbances and dementia. HD is an autosomal dominant disease, caused by a CAG trinucleotide repeat expansion in the *huntingtin* (*HTT*) gene, with a progressive loss of long projection neurons in the cortex and striatum [56]. Several mechanism and pathways have been proposed to explain the pathogenesis of HD, including transcriptional dysregulation, expanded polyglutamine repeat protein interactions with other proteins in the central nervous system, caspase activation, N-methyl-D-aspartate receptor (NMDAR) activation, calcium homeostasis abnormalities, abnormal mitochondrial bioenergetics and impairment in axonal trafficking [62-64].

The findings of mitochondrial abnormalities in brain or neuronal cells, such as impairment in respiratory chain complexes activities [62]; mitochondrial respiration and ATP production [65, 66], are important evidence of mitochondrial involvement in the pathogenesis of HD. Abnormalities in mitochondrial fusion and fission have recently been recognized as important factors in the pathogenesis and progression of HD [67]. Fragmentation of mitochondria and reduced mitochondrial fusion were associated with increased ROS in a study with cortical neurons treated with 3-nitropropionic acid (3-NP) [68]. 3-NP is an irreversible inhibitor of mitochondrial respiratory complex II, which induces HD-like pathology and symptoms in animal models. In this study, a dramatic rise in ROS induced mitochondrial fission and neuronal cell death. Furthermore, it was also demonstrated that over expression of proteins that stimulate mitochondria fusion can decrease the toxicity of mutated huntingtin protein in both cells and animals [69]. Additionally, defects in axonal transport, such as abnormal movement of mitochondria throughout the neuron, were also reported in cells or animal models and can contribute to the morphological defect associated with mutant huntingtin expression [42].

CONCLUDING REMARKS

There is a close relationship between mitochondrial dysfunction, generation of free radicals and neuronal death. Although the exact mechanisms of the pathogenesis of several diseases are not completely elucidated, great advances have been developed regarding the pathogenic pathways involving oxidative damage. With this knowledge, potential therapeutic approaches can be developed to increase antioxidant protection or restore mitochondrial function.

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CONFLICT OF INTEREST

The author(s) confirm that this chapter contents have no conflict of interest.

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Section 3: Most Frequent Neurodegenerative Diseases

CHAPTER 7

Alzheimer's Disease

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Abstract: Alzheimer's disease is the leading cause of dementia in the elderly. It is characterized by progressive memory loss and deterioration of cognitive ability, as well as by the presence of two main histopathological abnormalities in the brain: the amyloid plaques and the neurofibrillary tangles. The first is composed mainly of the aggregated form of the amyloid-beta peptide, while the latter consists of neuronal cell bodies filled with the hyperphosphorylated form of the tau protein. Although several genetic risk factors have been identified, the pathological mechanism of this disease remains elusive. As a consequence, to the present moment there is no cure to this condition or treatment capable of reliably reversing its symptoms. Hereditary forms of the disease typically have an early onset, and are predominantly associated with mutations in the molecular machinery responsible for the metabolism of a protein known as amyloid-precursor protein. In spite of the strong evidence suggesting its involvement in the pathogenesis of Alzheimer's disease, very little of the normal physiological role of this protein or its pathway is known. A second molecular pathway involved in many cases of neurodegenerative conditions, including Alzheimer's disease, is the cytoskeleton-associated protein tau. Tau plays an important role in biological processes like axonal transport, and much is known about the molecular mechanisms of tau dysfunction in disease. However, the precise mechanisms by which both amyloid and tau molecular signaling pathways interact in the pathology are not fully understood. As a result, the lack of a clear picture of the molecular alterations underlying this disease has represented a barrier to the development of effective treatments. In this regard, the two available options approved by the U.S. Food and Drug Administration target mostly the symptoms and provide unsatisfactory results in the long term. Many research groups in both academia and industry have focused efforts in the development of new therapies capable of reversing the cognitive impairment of patients with Alzheimer's disease. Several of the emerging therapies had severe side effects and disappointing outcomes in terms of improving cognitive levels. However, there are some therapies that have been showing more promising results. Further studies and clinical trials are still needed to fully address the risks and benefits of new treatments in Alzheimer's disease.

Keywords: Alzheimer's disease, amyloid, tau, treatment, Aβ, amyloid precursor

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protein, neurofibrillary tangles, amyloid plaques, neuroinflammation, memory, learning, memantine, glutamate, NMDA receptors, AMPA receptors, long-term potentiation, synaptic transmission, insulin, oxidative stress, excitotoxicity.

7.1. INTRODUCTION

Among the many forms of dementia that affect individuals in the late stages of life, Alzheimer's disease (AD) is the most common, with current estimates reaching more than 25 million cases globally (Alzheimer's Association, http://www.alz.org). Affected individuals typically experience memory loss, learning deficits, cognitive impairment and a myriad of emotional symptoms like depression and anxiety. This represents a very difficult situation for patients' families, as there is no cure at present, typically requiring long-term management of the symptoms, either at home or at institutions. Health care for AD patients imparts a major economic burden to society, with costs three- to four-times higher than for individuals without AD [http://www.alz.org]. A small (1 – 6%) number of cases can be attributed to inherited genetic causes, in which AD develops at early ages and is passed from one generation to another in a Mendelian fashion. These are called the familial forms of AD, and usually occur before the age of 65. However, most AD cases occur after 65 years and are weakly associated with a wide range of genes, the most common being the lipoprotein ApoE4 [1]. Given the high prevalence of AD among the elderly, age is considered the most important risk factor for developing AD [2]. Understanding the pathogenesis of AD has become particularly urgent because of the rapidly aging world population, and countless efforts have been made in this regard. However, after 100 years of its first clinical description, the causes of AD still remain controversial, and no effective treatment has been developed. In this book chapter, we will summarize the consensus of AD pathophysiology, and touch potential new avenues for drug discovery.

7.2. HISTORY

In 1906, the German pathologist Dr. Alois Alzheimer reported a case of early-onset dementia in a 51-year old woman named Auguste D., thus providing the first description of the disease that would later be named after him [3]. As pictured in her own words: "I have lost myself", this patient had experienced a great extent of memory loss and cognitive impairment. After her death, Dr.

Alzheimer autopsied her brain and found two intriguing microscopic abnormalities, which later turned out to be the characteristic pathological alterations of AD. One of them was the presence of large deposits of amorphous material in the space between cells, the "senile plaques". The other important finding was the presence of an anomalous flame-shaped mass within the cell bodies of many neurons, the so called "neurofibrillary tangles", which seemed to cause cells to atrophy and die. Many research groups have spent years characterizing the effects of the components of the senile plaques (also known as "amyloid plaques") and the neurofibrillary tangles in the brain, features that are still considered the main players in AD pathology.

7.3. PATHOLOGICAL MECHANISMS

7.3.1. Amyloid-β

The amyloid plaques are now well characterized as extracellular deposits of protein aggregate, surrounded by tissue showing signs of neuronal dystrophy, oxidative damage and inflammation. The major component of amyloid plaques is a peptide known as Amyloid-beta, or Aβ, which accumulates in the brains of AD patients. The discovery of Aβ generated an immense interest around the molecular mechanisms involved in its production, as well as its physiological role. As a matter of fact, Aβ is just a small fraction of a larger protein called APP (from Amyloid Precursor Protein), which is anchored in the plasma membrane of the cell. APP is the core of a complex metabolic pathway that is assumed to be involved in intercellular signaling. The proteolytic processing of APP by a series of enzymatic complexes called α–, β- and γ-secretases cuts APP into many different fragments in a regulated series of steps. These fragments, including Aβ, can be either secreted by the cell or engage in intracellular signaling. Thus, Aβ results from a proteolytic cascade involving multiple enzymes and associated by-products [4].

As evidence of how important the proteins involved in the Aβ pathway are to the pathophysiology of AD, virtually all mutations that cause hereditary forms of AD are involved in APP processing. The three main genes related to familial AD cases encode the proteins APP and presenilins 1 and 2 (both members of γ-secretase complex) [4]. Although the physiological functions of APP are still not fully understood, it is known to be essential for embryonic development. The

family of proteins to which it belongs is known to promote adhesion between cells, thereby regulating cell survival, neuronal adhesion and cell migration, and thus brain development [5].

That initial description of the amyloid plaques led scientists to think for many decades that the plaques were directly responsible for the clinical symptoms and neurodegeneration in AD. This idea developed into what is called the "classical amyloid hypothesis" of AD [6]. According to this hypothesis, Aβ triggered neurotoxicity, which caused neuronal death, ultimately leading to clinical symptoms. However, studies in transgenic animals that develop the amyloid plaques do not entirely corroborate the original hypothesis, as some of these animals display normal behavior [7]. Furthermore, many clinical studies have documented a poor correlation between the occurrence of clinical symptoms and presence of the amyloid plaques in post-mortem tissue [7]. In other words, not all patients who displayed symptoms of broad cognitive impairment characteristic of Alzheimer's disease actually have the plaques, and more importantly, amyloid plaques are found in the brains of people considered mentally healthy. Thus, a revised form of the original hypothesis was proposed [7].

Just when scientists were starting to realize that plaques might not be the only players in AD pathology, another piece of the puzzle was uncovered. Digging through a series of post mortem brain tissue from AD patients using biochemical techniques, researchers found soluble forms of Aβ aggregates, which could not have been observed with the techniques that were initially used to visualize the amyloid plaques. It was soon demonstrated that these forms were toxic to neurons, suggesting that it was not the end of the story for Aβ. The small and soluble forms of Aβ aggregates also build up and accumulate in the brains of AD patients, and are considered much more potent than plaques because, in experimental conditions, they can cause damage to neurons at much lower concentrations [8]. These soluble aggregates are called Aβ oligomers and are found in a range of different sizes and molecular weight, from small units containing only two monomers (the individual units of the Aβ peptide) up to larger ones [9].

In terms of diagnostics, some research initiatives have been trying to create methods to detect Aβ oligomers in the cerebrospinal fluid of patients, but no

effective technology has been developed so far. The greatest progress in this area came from recent advances in radiological imaging, which have enabled the detection of amyloid plaques in living patients with the use of compounds that label amyloid plaques. One of the most extensively investigated and validated tracer is Pittsburg compound B (PIB). After injection into the blood stream, PIB traverses the blood-brain barrier and binds to the amyloid plaques, due to its high affinity for fibrillar Aβ. PIB binding to amyloid plaques can be detected by positron emission tomography (PET), an imaging exam routinely employed in brain diagnostics. This technique has improved diagnostic accuracy and patient selection for clinical trials. Furthermore, it has the ability to detect amyloid plaques at very early stages, years before the manifestation of clinical symptoms [10]. Early detection might be used to prevent the development of symptoms once effective preventive measures become available.

7.3.2. Tau

The second major abnormality found by Dr. Alzheimer was the presence of the neurofibrillary tangles. These are found inside the cell body of neurons, whose axons and dendrites look dystrophic. Just like the amyloid plaques, the neurofibrillary tangles are made of aggregated protein, with the difference that a protein called Tau is their major constituent. Tau is normally found in the axons of neurons, where it associates with one of the components of the neuron's cytoskeleton, the microtubules. Its main function is to promote microtubule assembly and stabilization, which is important for normal axonal transport of vesicles and organelles.

In the brains of AD patients, Tau is found in an abnormal configuration: conjugated with an excessive amount of phosphate groups (hyperphosphorylated) and assuming a different three-dimensional conformation. While Tau is normally found as a soluble protein, this hyperphosphorylated form renders insoluble, filamentous aggregates that generate the neurofibrillary tangles [11]. The mechanisms responsible for the conversion of a normally soluble monomeric protein into the insoluble filamentous aggregates have been the subject of intense study [12]. It is well established that glycogen synthase kinase (GSK)-3β is the major enzyme that phosphorylates Tau. The sites of Tau phosphorylation by

GSK3β reside primarily within the microtubule-binding domain. When these sites are phosphorylated, the interaction between Tau and microtubules is disrupted. This finely regulated mechanism plays a physiological role in the long-term dynamics of synaptic function [11]. However, pathological GSK3β overactivation creates a permanent shift in the levels of phosphorylated tau, putatively leading to the dysregulation of this physiological mechanism and to harmful consequences for synaptic function. Furthermore, tau phosphorylation by GSK3β affects microtubule stabilization and dynamics. In the long run, hyperphosphorylation and aggregation of Tau can lead to microtubule disintegration and severely impair axonal transport and synaptic transmission, ultimately contributing to the behavioral deficits observed in AD [11]. Because GSK3β is constitutively active, its regulation is primarily based on inhibition of its activity through different signaling mechanisms, such as the insulin or wnt pathways [12]. It turns out that these two pathways can both be inhibited by high levels of Aβ [13]. In fact, GSK3β can be activated by fibrillar forms of Aβ, and active GSK3β is found in neurofibrillary tangles in postmortem AD brains [7]. These evidences suggest that GSK3β could be the link between Aβ and tau in AD.

The neurofibrillary tangles are better correlates of dementia in AD than the amyloid plaques, and thus could be considered the ultimate diagnosis criteria. On the other hand, neurofibrillary tangles are a key feature of many other neurodegenerative diseases which also result in dementia, such as frontotemporal dementia with Parkinsonism on chromosome 17 (FTDP-17) [11]. Nevertheless, all tauopathies share the common fact that neurofibrillary tangles are strongly associated with cell death, through mechanisms that involve activation of caspases, enzymes classically involved in a slow process of cell death. More recently, indirect evidence of the existence of Tau oligomers, smaller and soluble forms of Tau aggregates, in AD have arisen [11]. Although their existence is not confirmed, it would explain neurodegeneration observed in the absence of neurofibrillary tangles in an experimental model of tauophathy [11].

Overall, it is generally accepted that Tau is involved in AD pathology through mechanisms of hyperphosphorylation and aggregation, but whether neurofibrillary tangles themselves trigger cell death, or act as a buffer for a more toxic oligomeric tau, is still a matter of debate.

7.3.3. Synaptic Basis of AD Pathology

Does Aβ cause memory impairment in AD? This is perhaps the issue that has been most extensively approached experimentally in the field. Robust evidence in rodents and invertebrate models suggest that Aβ disrupts learning and memory, either when synthetic forms are applied exogenously, or in organisms that were genetically modified to produce increased amounts of the peptide [2]. Corroborating that idea is the fact that Aβ does actually affect the cellular and molecular mechanisms that underlie learning and memory [2].

In order to better understand how Aβ impairs learning and memory, it is important to have an idea of how the nervous system functions. Synapses are the points of communication between neurons, which form the basis for the complex functional networks in the brain. Most neurophysiological processes rely on synaptic transmission to occur. Briefly, upon the arrival of an electric signal, small molecules known as neurotransmitters are released from one neuron and perceived by the other through specific receptors lying on its surface. These receptors are proteins that have the ability to trigger another electric signal in the perceiving neuron, so that the information continues to propagate. There are different types of neurotransmitters, some being excitatory (as to facilitate propagation of electric impulse) and others inhibitory (as to stop this propagation). The integration of inhibitory and excitatory synapses in a given neuron is what determines functionality of a circuit, and both are important and have to work in a coordinate fashion. One key aspect of brain areas related to learning and memory is that synapses in these circuits are extremely malleable, or plastic. In other words, being plastic means having the ability to strengthen or weaken as a consequence of how much they are being activated. Thus, neuronal activity can modify neural networks by means of synaptic plasticity, a phenomenon essential for many neurological functions.

The major excitatory neurotransmitter in the brain is glutamate, and plasticity at glutamatergic synapses is believed to be crucial for learning and memory [14]. It is now widely accepted that Aβ disrupts glutamatergic synaptic transmission and prevents plasticity at these synapses [2]. Among the many mechanisms that are believed to be involved in the effects of Aβ, a disruption in the homeostasis of

intracellular calcium is perhaps the most important. Alters levels of calcium mediate the loss of glutamate receptors from the neuronal surface, alters release of glutamate from nerve terminals, and activation of many enzymes related to cell death [15]. The glutamate receptor subtype NMDA is believed to play a central role in the effects of Aβ, as it mediates the abnormal entry of calcium into the cell when Aβ is present. Some of the downstream effects of that calcium entry include the generation of reactive oxygen species (popularly known as "free radicals"), the reduction of glutamate receptors, and the loss of dendritic spines (membrane specializations where glutamatergic synapses are preferentially made). In fact, evidence suggests that NMDA receptors themselves could be targets of Aβ, which binds to neuronal membranes in a very specific way [16]. Altogether, these effects are believed to contribute to memory loss in AD, irrespective whether Aβ is the primary cause, or an intermediate step in the pathology. It is important to note, however, that the neuronal effects of Aβ are highly dependent on concentration, the ones reported above happening at pathological concentrations, in the order of high nanomolar to micromolar. In contrast, Aβ levels normally found in the healthy brain are extremely low, in the picomolar range, and may have opposite effects as those observed under pathological levels [17]. Indeed, studies have demonstrated that Aβ is normally released from axonal terminals and dendrites [18], and this occurs by means of neuronal activity [19, 20]. Furthermore, Aβ has been shown to be a positive regulator of presynaptic release of glutamate at excitatory synapses in the hippocampus, where it is also essential for the induction of activity-dependent forms of plasticity [17]. Altogether, these findings suggest that: in normal physiological conditions, Aβ is secreted during neurotransmitter release and acts as an important regulator of this process, ultimately playing a role in synaptic function; whereas in pathological states, the overproduction of Aβ results in synaptic dysfunction, leading to cognitive impairment.

Another important neurotransmitter that is greatly affected in AD is acetylcholine. Dysfunctional cholinergic transmission is thought to underlie, at least in part, memory impairment and cognitive deficits in AD [21]. Cholinergic neurons from the basal forebrain seem to be particularly susceptible since cholinergic dysfunction usually appears in early stages of AD, and there is an extensive loss of both cholinergic neurons and acetylcholine receptors in the late stages of the

disease. Additionally, there is a large body of evidence suggesting that the nicotinic subtype of acetylcholine receptors are targeted by Aβ, which induces abnormal activation of these receptors, followed by their internalization and loss of function [21]. The large extent of cholinergic dysfunction has important consequences in pathology and treatment, which will be discussed in the next section. Thus, it is generally accepted that cholinergic dysfunction is a major component of AD pathology.

7.4. THERAPEUTICS

Unfortunately, to date there is no available treatment capable of curing AD. However, there are medications that can help control the symptoms that may occur as the disease progresses. Currently, the only approved treatments for AD are the acetylcholinesterase inhibitors (AChEIs) and an N-methyl-D-aspartate (NMDA) receptor antagonist. We will discuss some important aspects of these two approved treatments, and then briefly comment on the most promising strategies that are currently being studied for the development of new treatments.

7.4.1. Approved Treatments for AD

7.4.1.1. Acetylcholinesterase Inhibitors (AChEIs)

Patients with AD have low levels of the neurotransmitter acetylcholine, an important brain chemical involved in nerve cell communication. AChEIs were the first class of drugs ever used to treat AD and are indicated for the mild and moderate stages of the disease. These drugs increase the availability of acetylcholine at the synapses by preventing its breakdown by the enzyme acetylcholinesterase. This enables acetylcholine to work for a longer period of time, interact with cholinergic receptors, and affect the uptake, synthesis, and release of neurotransmitters.

The AChEIs approved for mild to moderate symptoms of Alzheimer's disease by the U.S. Food and Drug administration (FDA) are donepezil (Aricept®), rivastigmine (Exelon®), galantamine (Razadyne®), and tacrine (Cognex®). Aricept® is also approved for severe Alzheimer's symptoms. Some drugs might display effects beyond the inhibition of acethycholinesterase: Galantamine also

modulates nicotinic acetylcholine receptors, and rivastigmine inhibits butylcholinesterase, but the importance of these additional properties is unknown. Meta analyses have repeatedly found that AChEIs have a modest beneficial effect on cognition and memory [22].

AChEIs are the first choice of treatment for AD, but they present some limitations, such as elevated cost, modest benefits, and short period of effectiveness. Furthermore, most AChEIs have considerably short half-lives, and may cause side effects, such as nausea, vomiting, diarrhea, weight loss, and dizziness, resulting from activation of peripheral cholinergic systems [23].

7.4.1.2. N-methyl-D-aspartate (NMDA) Receptor Inhibitor

Damage from excitatory amino acid neurotransmitters, especially glutamate, can produce excitotoxicity and cell death [24]. The receptor mostly involved in excitotoxicity is the NMDAR. If NMDAR sites are overactivated, high levels of Ca^{2+} can enter the cell, creating reactive oxygen species and activating specific enzymes involved in cell death. Importantly, however, normal NMDA receptor activity mediates, in large measure, physiological excitatory synaptic transmission in the brain and is therefore crucial for the normal functioning of the nervous system.

In AD it is well characterized that there is an excessive stimulation of glutamate receptors, especially of the NMDA subtype. Therefore, antagonists of these receptors have held much promise for an effective treatment. Most clinical trials involving NMDA receptor antagonists have failed due to unwanted side effects induced by the blockage of the normal glutamatergic function. Interestingly, a drug called memantine was the first in a novel class of AD medications to act on the glutamatergic system. Memantine selectively blocks only excessive stimulation by binding to NMDA receptors, suppressing the influx of Ca^{2+} and resulting in a small improvement in cognition and behavior [24-26].

Memantine is marketed under the brands Axura® and Akatinol® by Merz Pharmaceuticals, Namenda® by Forest Laboratories Inc., Ebixa® and Abixa® by Lundbeck and Memox® by Unipharm Inc. While the AChEIs only treated the symptoms of mild to moderate AD, Memantine (Namenda®) was the first drug

approved in 2003 by the FDA to treat the symptoms of moderate to severe AD. Overall data suggest clinically significant effects on cognition, mood, and performance of daily activities in more severe cases of AD [27-30] and there is little evidence of effect in mild cases of AD [31, 32].

Memantine is generally well tolerated; the most common side effects are back pain, constipation, diarrhea, dizziness, drowsiness, headache, pain, and weight gain. Conversely, despite being a neuroprotective drug, memantine can have neurotoxic effects. A study performed in an animal model demonstrated that the simultaneous use of donepezil (an AChEIs) and memantine produced a substantial increase in neurotoxic reactions [33]. While evidence of such toxicity in humans is still preliminary, the simultaneous use of both drugs demands caution. Conversely, recent studies show that the combination of an AChEI and memantine slow cognitive decline more efficiently than any of these drugs alone, and the benefits of the combination therapy seem to persist for years [34].

7.4.2. Emerging Therapies

7.4.2.1. Therapies Against Aβ

According to the amyloid cascade hypothesis, Aβ has a pivotal role in the pathogenesis of AD [35]. The key step regulating Aβ generation is the sequential proteolytic processing of APP by β-secretase (BACE) and γ-secretase proteases. When the α-secretase pathway is used for APP processing instead, no Aβ is produced. Gamma- and β-secretases cleave APP in two different spots, separating Aβ from its progenitor. These peptides can aggregate into oligomers, and these oligomers can clump together to form larger plaques. In every step of the aggregation process there are opportunities to control the disease.

One possible approach involves inhibiting γ- and β-secretases. If these enzymes can be prevented from cleaving APP in the first place, there is no generation of Aβ, and consequently no toxicity. But targeting these enzymes has proven to be tricky, partly because they are not specific to APP, being able to cleave other proteins as well. There are 40+ endogenous substrates for γ-secretase whose cleavage may play important roles in the adult human, most notably Notch [36]. Beta-secretase also cleaves numerous other substrates with important

physiological activity, including neuregulin-1, which is involved in myelination [37].

Many research groups in both academia and industry have synthesized γ- and β-secretase inhibitors in the hope of developing a therapy able to attenuate the abnormal production of Aβ and to halt or even reverse the progression of AD. The drug semagacestat (LY450139) – that blocks γ-secretase – was a candidate drug to treat AD [38]. It was originally developed by Eli Lilly & Company and Élan Corporation plc, with clinical trials conducted by the former. Phase III trials included over 3000 patients, but in August 2010 a disappointing interim analysis had to put an end to the tests: patients treated with semagacestat did significantly worse in cognitive assessment and activities of daily living than did subjects in the placebo group. Furthermore, semagacestat is associated with an increased risk of skin cancer compared with those who received placebo.

Beta-secretase inhibitors have been shown to reduce Aβ in animal models and may have fewer adverse effects than γ-secretase [39, 40]. The big challenge has been to engineer a molecule that is large enough to inhibit the beta-secretase's active binding site but in the same time small enough to pass through the blood–brain barrier. Due to the complicated inherent chemistry issues, only one compound (CTS-21166) has proceeded to clinical testing [41]. Presently, most research involves developing secretase inhibitor molecules that will penetrate the blood-brain barrier, produce beneficial results, and not produce adverse effects.

Among all of the approaches targeting AD, the most exciting and advanced is the Aβ-targeted immunotherapy. There are two different approaches to generate antibodies directed against Aβ: one is active immunization with intact $A\beta_{1\text{-}42}$ and small fragments of Aβ conjugated to an unrelated carrier protein, and the second is passive immunization administering anti-Aβ antibodies directly [37, 42-44]. Using both approaches, Aβ levels in the brain were substantially reduced in phase I trial study [45].

An abundance of preclinical studies suggest that immunization with $A\beta_{1\text{-}42}$ is able prevent or reverse the development of AD pathology [46]. However, a phase II clinical trial initiated in 2001 in patients actively immunized with the drug

AN1792 was abruptly terminated in January 2002 due to the development of serious side effects, as aseptic meningoencephalitis and leukoencephalopathy, in 6% $A\beta_{1-42}$ vaccinated patients [47, 48]. Despite this trial termination, follow up studies of the trial participants have shown that AN1792 immunization increased the clearance of amyloid plaques [49].

Building upon lessons learned from the AN1792 trials, many groups from both academia and industry have generated novel active and passive Aβ vaccines. Two potential examples of monoclonal antibodies against $A\beta_{1-42}$ peptide are solanezumab from Eli Lilly & Company and bapineuzumab from Janssen Pharmaceuticals and Pfizer, Inc. (originally developed by Élan Corporation plc). Both drugs were tested in phase III trials on thousands of participants with mild-to-moderate AD. However, in 2012, bapineuzumab failed to produce significant improvements in cognitive or functional performance compared with placebo in patients who did not carry a variation of a gene of apolipoprotein (ApoE) ε4, a reported risk factor of AD [1, 50]. In October of 2012, results from the phase III trials with solanezumab suggested it might modestly slow mental decline, especially in patients with mild disease. Overall, these studies missed their main goal of significantly slowing the development of disease or improving activities of daily living. Although promising, the results with solanezumab did not meet the criteria to win FDA approval. For this reason, Eli Lilly & Company is planning to start another large study to confirm the results with solanezumab treatment in patients with mild AD.

Despite having showed promising results in animal models of AD, clinical trials with Aβ-targeted immunotherapies have had rather disappointing outcomes. One possibility is that treatments that target Aβ should be administered before the onset of clinical symptoms, or only during the mild cognitive impairment stage (not moderate or even severe stages) [51, 52]. This might explain the failure of past immunotherapy trials, as antibodies were administered after the development of full-blown AD.

7.4.3. Therapies Targeting Tau

Tau research has progressed more slowly than research on Aβ. Partly, this happened because of limitations in funding and the overwhelming focus in Aβ. Another factor that likely contributed is the difficulty in studying proteins that,

like Tau, are essential to maintain the health of cells. As described in the previous section of this chapter, Tau associates with tubulin to stabilize axonal microtubules during its polymerization in healthy neurons. In AD, however, Tau acquires too many phosphate groups, aggregates, and becomes dysfunctional. This process results in microtubule collapse and the block of neuronal signaling. Thus, inhibition of Tau hyperphosphorylation and promotion of filament disassembly represent two viable strategies for disease-modifying therapies [32, 53].

One main target in the Tau phosphorylation pathway is GSK-3β, which is up-regulated in the frontal cortex of AD patients [54], and has been associated with an increased risk of AD [55]. The ion lithium, which is regularly prescribed to patients with bipolar disorder as lithium carbonate, is an inhibitor of GSK-3β. Therefore, it could represent a potential therapeutic strategy in AD. In fact, in a case-control study, bipolar geriatric subjects taking lithium had a decreased risk of developing AD over a 6-year period compared with an age-matched group not taking lithium [56]. However, a phase II clinical trial performed in 71 patients with mild AD treated with lithium for 10 weeks was disappointing, not having met clinical or biomarker efficacy [57]. Further clinical trials in larger populations are warranted in order to further evaluate the effects of lithium in the treatment of AD.

7.4.4. Statins

Despite numerous studies connecting lipid metabolism to AD pathogenesis, relatively few therapeutic approaches have exploited this connection thus far, with the exception of drugs affecting cholesterol metabolism, such as statins. Disordered cholesterol metabolism is a common feature in many risk factors associated with AD [58]. These include hypercholesterolemia, coronary artery disease [59, 60], and cerebrovascular disease [61, 62].

The biochemical pathway for cholesterol synthesis in all animal cells involves a key enzyme known as HMG-CoA reductase. This enzyme has the important function of catalyzing the convertion of 3-hydroxy-3-methylglutaryl-CoA into mevalonic acid, which is crucial to generate cholesterol. By competitive inhibition of HMG-CoA reductase, statins reduce the production of cholesterol and the intermediate products called isoprenoids.

In addition to reducing cholesterol levels, statins might also influence Aβ production and aggregation through other biological mechanisms [63-65]. Increasing evidence suggests that inflammation can play an important role in the pathogenesis of AD through the activation of microglial cells [8]. Statins can have anti-inflammatory effects on macrophagic cell lines, including the downregulation of MHC class II molecules [66]. The second evidence is that Aβ might have cytotoxic effects on neurons and oligodendrocytes by releasing free radicals from activated microglia and astrocytes [67]; as statins inhibit nitric oxide (NO) production and inducible nitric oxide synthase (iNOS), they might improve the harmful effects of free radicals [68]. Lastly, statins were shown to reduce the expression of ApoE, the principal cholesterol carrier in the brain [69].

Retrospective case control studies suggest that statins reduce the risk of developing AD, but clinical trials and prospective cohort studies have produced inconsistent, mixed results [70, 71]. Further studies are clearly needed to fully address the risks and benefits of statins use in AD.

7.4.5. Anti-Inflammatory Drugs

Neuroinflammation contributes to neuronal damage in the brain and is implicated in AD pathogenesis. The presence of activated microglia and inflammatory substances such as cytokines and cyclo-oxygenase (COX)-2 enzymes has been associated with plaques and tangles found in AD. This field of research initially gained a great amount of attention due to the interest in the potential ability of anti-inflammatory drugs to prevent AD pathology progression. One study performed in 49,349 individuals, who had used nonsteroidal anti-inflammatory drug (NSAID) for at least five years, demonstrated that the risk of acquiring AD was decreased. Ibuprofen and Indomethacin had the greatest effect, while Celecoxib and the Salicylates offered no protection [72, 73]. However, up to now, the clinical trials investigating the disease-modifying potential of anti-inflammatory drugs have been unsuccessful.

7.4.6. Intranasal Insulin Treatment

Insulin is critical for normal brain function, and abnormal insulin metabolism has been shown to contribute to the development of AD. Brain levels of insulin and its

receptor are significant reduced in AD patients. Furthermore, insulin signaling was found to be impaired in both postmortem analysis of patients' brains and in animal models of AD [74, 75]. Brain insulin has been pointed as a key component of learning and memory [76, 77], suggesting that insulin resistance may contribute to cognitive impairment seen in AD. These findings suggest that impaired brain insulin signaling plays a critical role in the etiology of this disease and it has been hypothesized that raising these levels to normal might help maintain cognitive ability.

Therapeutically, insulin represents an interesting target, as it is known that when administered intranasally to humans, insulin bypasses the bloodstream to reach the cerebrospinal fluid (CSF) within 10 min without substantial absorption into the circulation [78]. Furthermore, studies have demonstrated that such form of administration of insulin improves cognitive measurements in healthy subjects [79-81], highlighting intranasal insulin as a candidate treatment for AD.

Pilot studies [82, 83] and a clinical trial [84] in patients with early to mild AD treated with intranasal insulin showed promising results with respect to cognitive improvement. The clinical trial consisted of 4 months of intranasal insulin treatment and the results showed that the ability to memorize episodic items was preserved in insulin-treated AD patients, compared to placebo. Interestingly, this episodic memory-preserving effect was also observed two months after completion of the insulin treatment [84]. Importantly, no significant side effects of intranasal insulin administration were reported.

While these are promising results, caution to interpret these findings is necessary. The observed effects were subtle and the period of treatment was relatively short compared with the duration of the disease. Therefore, further studies are still required to determine the clinical relevance of intranasal insulin treatment.

7.4.7. Pleiotropic Treatment: Cerebrolysin

The development of drugs with pleiotropic activity (*i.e.*, acting at different levels of the AD pathogenic process) seems to constitute another promising approach. One example of a pleiotropic treatment in AD is the drug called Cerebrolysin – a neuropeptide preparation that mimics the action of endogenous neurotrophic

factors. Several *in vitro* and *in vivo* studies have shown that Cerebrolysin has neurotrophic effects similar to those of endogenous neurotrophic factors, supporting the survival, stability, and function of neurons.

Several randomized, double-blind, clinical trials using Cerebrolysin showed reliable benefits in the overall clinical function and cognition, improvements in behavior, and minor effects on daily living activities in patients with mild to severe AD [85]. While it is approved for the treatment of AD in 44 countries worldwide, being available in an intravenous form with good tolerability, it has not yet been approved in the United States. Further studies with Cerebrolysin, including longer term trials and exploration of its use in combination with AChEIs, are needed to more clearly determine its place in the treatment of AD.

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CONFLICT OF INTEREST

The author(s) confirm that this chapter contents have no conflicts of interest.

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CHAPTER 8

Parkinson's Disease: Genetics, Mechanisms and Diagnosis

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Abstract: Parkinson's disease is a complex neurodegenerative disorder, mainly characterized by the loss of dopaminergic neurons in the *substantia nigra* and their projections to the *striatum*, causing several motor deficits. Neuronal cytoplasmic inclusions, named Lewy Bodies, are found in the affected areas. Parkinson's disease is distributed worldwide, affecting all ethnic groups and socioeconomic classes. Protein homeostasis is crucial for preventing neurodegeneration. Misfolding of proteins can lead to loss or gain of function, resulting in protein dysfunction and causing various types of diseases. Five genes containing pathogenic mutations were identified to contribute for incorrect protein conformation in Parkinson's disease. Mitochondrial dysfunction and purinergic receptor signaling are also involved in the mechanism of disorder. Several types of pharmacological intervention were developed. Dopamine agonists are the most common therapeutic agents used currently. *N*-methyl-D-aspartate type glutamate receptor antagonist, monoamine oxidases and anticholinergic drugs can be therapeutic alternatives. New techniques and studies have contributed to the discovery of new genes and genetic risk factors for Parkinson's disease. Brain banks and imaging analyses can also be very useful tools for understanding the mechanisms of disease progression. Current studies on molecular aspects of Parkinson's disease, together with the development of new drugs, techniques and tests to improve diagnosis accuracy will bring new perspectives for PD therapies.

Keywords: Parkinson's disease, genetic mutations, pharmacology, protein misfolding, oxidative stress, dopamine, L-DOPA, purinergic receptors, P2X7R, PPAR, PGC-1α, α-synuclein, LRRK2, PARKIN, animal models, epidemiology, diagnosis, therapy, ubiquitin-proteasome system, imaging.

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8.1. BACKGROUND

Parkinson's disease (PD) is a complex neurodegenerative disease, whose etiology is still unknown. It is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta* and their projections to the striatum, causing several motor deficits. However, changes are not restricted to the substantia nigra and may be present in other brain stem nuclei (*e.g.*, dorsal motor nucleus of the vagus), cerebral cortex and peripheral neurons [1]. The presence of degenerative process beyond the nigrostriatal system may explain a number of non-motor symptoms and signs present in PD, such as olfaction impairment, sleep disturbances, postural hypotension, constipation, emotional changes, depression, anxiety, psychotic symptoms, cognitive loss, *etc*. [2]. Neuropsychiatric alterations and cognitive decline may also occur at early stages of PD. PD was first described as shaking palsy by James Parkinson in 1817. Besides dopaminergic neuronal degeneration, the presence of neuronal cytoplasmic inclusions in *substantia nigra*, *locus ceruleus*, amygdala and the CA2 area of the hippocampus [3] from patients was observed *post-mortem*. These inclusions were named Lewy Bodies (LB) in 1920 by Frederick Lewy. Rolf Hassler, in 1938, showed that the *substantia nigra* was the main cerebral area affected, which was already suggested by Konstatin Tretiakoff in 1919. Identification of deficiency in the neurotransmitter dopamine in *substantia nigra* was made in 1950 by Arvid Carlsson. It was only in the 60s, after the identification of pathological and biochemical changes in the brain of PD patients, that levodopa (L-3,4-dihydroxyphenylalanine, L-DOPA) was introduced, which represented a major advance in PD therapy. L-DOPA is a naturally occurring amino acid, found in young vegetables, like various types of beans. L-DOPA is the precursor of the catecholamines dopamine, norepinephrine and epinephrine. While dopamine itself is not able to cross the blood-brain barrier, L-DOPA is. After entering the central nervous system, it is converted into dopamine by aromatic L-amino acid decarboxylase, also called DOPA decarboxylase (DDC). Using inhibitors of the dopamine converting enzymes, L-DOPA is able to reach the brain and subsequently be converted to dopamine, which is released in the synaptic cleft. L-DOPA has a 90-minute half-life, and this can be a problem because it leads to a peak of dopamine release in the synaptic cleft [4]. Clinical benefits were observed for virtually all patients, reducing the mortality in PD. However, complications after long-term

treatment, like adverse effects, including motor fluctuations, dyskinesia and neuropsychiatric complications, became apparent [5, 6].

8.2. EPIDEMIOLOGY

Even though diagnosis of PD is still imprecise, it is estimated that around 750,000-1 million people in the USA and 120,000–130,000 in the UK have PD, and the number of new cases is influenced by diagnostic accuracy. Members of the Kaiser Permanente Medical Care Program of Northern California health maintenance organization performed a study of age/gender and ethnicity-related PD incidence, evaluating 588 newly diagnosed PD patients. They observed that PD incidence in men is 91% higher than in women and also increases with age. Also, this age/gender-adjusted rate was highest for Hispanics, followed by non-Hispanic Whites, Asians and Blacks. Among Asians, PD incidence was slightly lower among men than women, but in the other groups, PD incidence was twofold higher among men than woman [7].

PD is a disease of worldwide distribution and affects all ethnic groups and socioeconomic classes. It is estimated that the worldwide annual cost with antiparkinsonian drugs is around $ 11 billion, which is about 3 to 4 times more expensive for patients in the advanced stage of the disease [8, 9]. A prevalence of 100 to 200 cases per 100,000 in habitants is estimated. The incidence and prevalence increase with age [10], and the average age of onset of symptoms is approximately 60 years. In young-onset PD, the onset occurs between 20 and 40 years old, and in juvenile-onset, PD starts below 21 years of age [11]. The risk of men to develop PD is about 1.5 times greater than that for women. PD is more common in Caucasians, less common in West Africa, and intermediate in China. The fact that African and Chinese descendants born in America have higher rates of PD than their counterparts in West Africa or China, suggests the role of environmental factors. The estimated standardized mortality rate, *i.e.*, the ratio of number of deaths in PD patients compared to controls, varies between 1.5 and 2.4. PD is not usually a direct cause of death, but a contributory cause in only half of the death certificates in PD patients. The primary cause in general is a complication such as infection. Studies demonstrated that tremor-dominant patients have better survival compared to those without tremor and / or with

postural instability and gait disturbance. These observations can be explained, at least partially, by the pathological findings showing neurodegeneration in different areas in tremor-dominant *versus* akinetic–rigid type patients. Another explanation may be errors in diagnosis. Some tremor patients may not have true parkinsonism and have better survival, and akinetic–rigid patients may have a rapidly progressive Parkinson-plus syndrome. Estimates from imaging studies show that symptoms appear when at least 50% of striatal dopamine is depleted and 60–80% of neurons in the *substantia nigra* are lost. Usually, a dose-related improvement in the motor function score is observed when patients initiate therapy with L-DOPA. Side effects, such as dyskinesias, traditionally occur in 50% of patients after 5 years on L-DOPA. In general, 70% patients on monotherapy with dopamine agonists remain free of dyskinesia after 5 years. However, measurements of motor improvement by the unified PD rating scale (UPDRS) showed L-DOPA is more effective than dopamine agonists [12]. Several studies suggest that the rate of decline is more rapid initially, and then slows in the more advanced disease stages, possibly due to treatment effects that influence severity and progression data [4].

8.3. GENETIC COMPONENTS

Although most of PD cases are sporadic, around 10% of PD patients have a family history compatible with a monogenic inheritance. There is no clinical symptom to distinguish between the sporadic and familial forms, familial PD patients are younger than those with sporadic form, at the onset of the symptoms [13]. The identification of novel genetic mutations, as well as the study of the effects of these mutations in familial PD, can also be relevant to understand sporadic PD. In the present scenario of genetic research, five genes containing pathogenic mutations were identified. Two of them are autosomal dominant, α-synuclein (SNCA) and leucine-rich repeat kinase 2 (LRRK2), and the other three underlie autosomal recessive disease, PARKIN, DJ-1 and PTEN-induced putative kinase 1 (PINK1) [13]. Another gene, ubiquitin carboxyl-terminal esterase L1 (UCHL1), also known as PARK5, seems to be involved in PD, but its function and importance are still being evaluated. Several studies have reported the roles that these genes play in disease development when mutated. Alterations in protein

phosphorylation and protein accumulation are some of the phenomena observed in studies of genetic mutations.

8.4. MISFOLDING OF PROTEINS

Protein homeostasis is a crucial factor in preventing neurodegeneration. Maintaining the correct conformation of a protein is necessary for it to exert its functions normally. Incorrect conformations of proteins can lead to loss or gain of function, resulting in protein dysfunction and causing various types of diseases. The role of chaperones in conformational diseases was first shown in trinucleotide repeat expansion diseases, such as Huntington´s and spinocerebellar ataxias [14]. Chaperones are specific proteins responsible for the correct folding of newly synthesized proteins and also in protein refolding, to correct erroneous protein folding [15]. Some chaperones, called heat shock cognates (HSCs), are responsible for correct folding in normal cellular conditions. However, under conditions of cellular stress, other chaperones, the heat shock proteins (HSPs) are expressed and activated [16]. It has been shown that heat-shock proteins (HSPs) have an important role in neurodegenerative diseases [17, 18]. In addition, chaperones and co-chaperones are also involved in degradative pathways, such as ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP) [19], thus removing the proteins irreversibly ill reeled. UPS is involved in both familial and in sporadic PD [20-22]. The UPS includes an ubiquitin activator (E1), an ubiquitin conjugator (E2) and an ubiquitin ligase (E3). After ubiquitin addition, target substrates are finally degraded by the proteasome [23]. PARKIN is an E3 ubiquitin-protein ligase [24] that mediates its own ubiquitination and also of other proteins, such as α-synuclein-interacting protein synphilin-1, endothelin-associated parkin-like receptor (PaelR), cyclin E, α and β tubulin, and the p38 subunit of the aminoacyl-tRNA synthetase complex (p38/JTV-1) [25]. Recent studies suggest that up to 50% of hereditary PD, and 10% of PD cases with early onset are linked to mutations in the PARKIN gene [26-28]. For these reasons, chaperones and co-chaperones have been investigated for the development of new therapies for PD [29]. α-synuclein, is a 140 amino acids protein, belonging to the family of β and γ-synuclein and synoretin, and it is highly expressed in both glial and neuronal cells in regions of the neocortex, CA2 and CA3 regions of the hippocampus and in the *substantia nigra* of adult brain, especially in presynaptic terminals [30-32]. The function of α-synuclein in the healthy brain is not yet fully known, but several studies show the accumulation and

fibrilization of this protein in LBs. This fact can be observed in cases of PD with multiplication of the SNCA gene [33-35]. The fact that the accumulation of α-synuclein is specifically deleterious to DA neurons appears to be due to the stabilization of oligomeric intermediates of α-synuclein by dopamine secreted by DA neurons [36]. The formation of oligomers, fibers and small aggregates of α-synuclein are initial processes that occur in neurodegeneration in PD. Misfolding of α-synuclein and its subsequent aggregation is prevented by the action of chaperones. Several studies have confirmed the presence of Hsp70, Hsp40 and Torsin in LB [37-40] revealing a possible cellular tentative to control protein aggregation. These studies are supported by the fact that up-regulation occurs directly to Hsp70, Hsp40, Hsp27 and in response to over expression of α-synuclein in mouse model of PD [41]. PARKIN is a 465 amino acid protein with characteristics domains of E2-dependent E3 ubiquitin ligases (E3s), which are involved in the addition of polyubiquitin chains to proteins that will be subsequently degraded by the UPS [24]. Mutations in the PARKIN gene are found in 50% of cases of recessive juvenile parkinsonism with early onset (<45 years) and about 77% of sporadic cases with onset below 20 years [27]. The mutations found in the PARKIN gene cause several alterations in its ubiquitination properties [42]. Some targets for ubiquitination by PARKIN are synphilin-1 and a glycosylated form of α-synuclein [43]. In cases of mutations that lead to inactivation of PARKIN, ubiquitination does not occur and the accumulation of atypical proteins results in toxicity to DA neurons [44]. A mutation in the UCHL1 gene, located on chromosome 4p, was identified in a family of German origin [44] and although no other mutation in this gene has been reported, a polymorphism was associated with sporadic PD in several studies [45, 46]**.** Searching for substrates of the kinases LRRK2, PINK1 and PARKIN, which directly affect the phenotypes observed in PD, have been the limiting step for advancing in research. A possible substrate of the kinase activity of LRRK2 is the 4E-binding protein (4E-BP) [47], a negative regulator of EIF4E (eukaryotic translation initiation factor 4E) which in turn plays a role in regulating protein synthesis, especially under conditions of cellular stress. Phosphorylation of 4E-BP diminishes its binding to eIF4E, increasing translation. It has been already shown that over expression of 4E-BP in Drosophila protects neurons against the toxic effects exerted by LRRK2 mutants [47]. Whether 4E-BP is a direct target of LRRK2 in PD is still not possible to confirm, since studies showed that 4E-BP is not an excellent substrate of LRRK2. Other kinases, such as p38, are much more effective in

phosphorylating 4E-BP than LRRK2. DJ-1 is involved in response to oxidative stress, but its importance in the DA neuronal death is still unclear.

8.5. OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION

Among the causes of neuronal death, free radicals are one of great importance. Although free radicals exist in normal cellular metabolism, in oxidative stress conditions, they may have deleterious effects on DNA and influence gene expression by modulating intracellular signaling pathways, leading to a diversity of diseases. In normal conditions, there are scavenging systems present to protect respiring cells from the adverse effects of free radicals. Reduced level of the antioxidant glutathione was found in PD, indicative of oxidative stress. Maintenance of certain levels of reactive oxygen species (ROS) and other free radicals is necessary for the normal physiology of living organisms. Mitochondria are largely responsible for ATP production by oxidative phosphorylation [48], and most mitochondrial diseases are caused by the impairment of ATP synthesis [49]. Therefore, tissues requiring high energy, such as muscle and brain, are most affected by bioenergetic changes [49]. In PD, accumulation of iron in the substantia nigra favors the formation of free radicals. Also, animal models of PD derived from MPTP administration, have a defective mitochondrial function in DA neurons. MPTP (1-methyl-4-phenylpyridine) is highly lipophilic and able to cross the blood-brain barrier within minutes [50]. The pro-toxin MPTP is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by monoamine oxidase B (MAO-B) in astrocytes and serotonergic neurons, the only cells that contain this enzyme. It is then converted to MPP+, probably by spontaneous oxidation, which is the active toxic molecule, and released into the extracellular space. MPP+ is a high-affinity substrate for the dopamine transporter (DAT), as well as for norepinephrine and serotonin transporters [51, 52].

In a similar manner, some pesticides may trigger the inhibition of complex I, a component of the respiratory chain, also known as NADH coenzyme Q reductase, which transfers the electrons to coenzyme Q. As a result of complex I inhibition, mitochondrial respiration decreases and allows leakage of free radicals. Failure in mitochondrial respiration is closely related to oxidative stress, although it remains to be clarified which of these processes comes first [4].

The *peroxisome proliferator-activated receptors* (PPARs) belong to a subfamily of nuclear receptors, which regulates the expression of genes involved in metabolic pathways. PPAR-α is expressed in neurons and astrocytes and also in macrophages and endothelial and smooth muscle cells. PPAR-α regulates negatively the transcription of NF-κ-B, inhibits inducible nitric oxide synthase (iNOS) in macrophages and blocks cyclooxygenase-2 (COX-2) expression in smooth muscle cells, inhibiting the inflammatory responses. PPAR-α also stimulates the expression of catalase and superoxide dismutase, antioxidant enzymes involved in the process of free radical elimination. Genes expressed in response to the *peroxisome proliferator–activated receptor γ coactivator-1α* (PGC-1α) are less abundant in PD patients. Recently it was demonstrated that activation of PGC-1α blocks the loss of DA neurons in cellular models of PD [53] and is able to prevent the damage caused by inflammation in Amyotrofic Lateral Sclerosis (ALS) [54]. Therefore, activation of PGC-1α may be a new therapeutic target in the treatment of diseases related to mitochondrial alterations [55-59].

8.6. PURINERGIC RECEPTOR INVOLVEMENT

P2 receptors are classified into two families, ionotropic P2X and metabotropic P2Y receptors. P2X receptors are ATP ion channels, and their agonists include ATP and its derivatives α,β-meATP and BzATP. P2X receptors are expressed in a wide variety of cell types, like neurons, heart and skeletal muscle, smooth muscle, leukocytes and platelets. P2Y receptors are present in almost all human tissues. They are G-protein coupled receptors that bind to ATP, ADP, UTP, UDP, NAD+, NAADP+, among others [60].

To date seven P2X receptors have been cloned (P2X1-7) and twelve P2Y (P2Y1, P2Y2, P2Y4-6, and P2Y8-14) from mammals and birds [61, 62]. It has been recently shown that P2X7 receptors (P2X7R) are overexpressed in animal models for many neurodegenerative diseases, suggesting P2X7R involvement in neurodegeneration. Prolonged exposure of P2X7 agonist leads to formation of large pores in the plasma membrane, enabling the uptake of large molecules and causing cell death [63, 64]. P2X7R plays an important role in the degeneration of DA neurons in PD *via* disruption of ion homeostasis and release of interleukin-1β [65, 66]. In the central nervous system, P2X7R are present in microglia and

astrocytes, but probably these receptors exist also on damaged DA neurons, since, after stimulation of P2X7R, it is possible to find cytosolic pores in the membrane of DA neurons. Using P2X7R antagonists caused improvement in distinct animal models for neuropathologies. Blocking the activity of P2X7R in spinal cord astrocytes reduced neuroinflammation in an ALS model [67]. These data suggest the possibility of using blockers P2X7R as therapies for neurodegenerative diseases [68].

8.7. NEW PERSPECTIVES FOR GENETIC RESEARCH IN PD

GWAS and NGS (genome-wide association scan and next generation sequencing, respectively) consist of new approaches that help to discover new genetic factors involved in PD. Studies of genotype-phenotype correlation have been made to characterize earliest signs of the disease. The GWAS technique allows the association of risk factors for diseases of high complexity, through large scale population-based studies. In 2011, investigators from the International Parkinson's Disease Genomics Consortium (IPDGC) identified 11 loci for genome-wide significance, six of which had been previously identified (MAPT, SNCA, HLA-DRB5, BST1, GAK and LRRK2), and five new (ACMSD, STK39, MCCC1/LAMP3, SYT11 and CCDC62 / HIP1R). The second study of IPDGC, in collaboration with the Wellcome Trust Case Control Consortium 2, identified five other PD risk loci (PARK16, STX1B, FGF20, and STBD1 GPNMB) [69, 70]. Marder and colleagues found that the susceptibility to develop PD is more than twice higher in first-degree relatives of PD patients than in first-degree relatives of control individuals. Moreover, a relationship between gender and ethnicity was also observed. Men and Caucasians seem to be at a higher risk than women and African and Hispanics are, respectively [71]. Most attempts to identify the susceptibility genes in sporadic PD have followed a candidate gene approach. Based on pathological, biochemical and epidemiologic findings, hypotheses on the etiology of PD can be generated, and genetic polymorphisms within gene that are thought to be involved in these pathways have been examined. Unfortunately, no consistent findings have emerged so far. Risk factors can be potential targets for novel treatments. A multicenter study investigated the association of 121 exonic variants in LRRK2 in PD with more than 15000 Individuals of different ethnic backgrounds. The results from this study added new information to the

GWAS, showing that different variants of the same gene may have independent effects on the risk for PD [72]. Mutations in the glucocerebrosidase (GBA) gene have an intermediate frequency in PD patients (6.7% in a large European study) [73], and those carrying the heterozygous mutations in GBA may be at higher risk of developing cognitive impairment [74]. Despite the numerous studies of genetic mutations and risk factors involved in PD, applying a genetic approach to achieve tailored medicine is still far from being reached.

8.8. BRAIN BANKS AS TOOLS FOR UNDERSTANDING PD MECHANISMS

Brain banks are vital in the scientific research of PD because they have the facilities and the expertise to recruit, classify, preserve and distribute specimens for research, abiding by the local ethical and legal framework [75-77]. *Post-mortem* brain tissue, cerebrospinal fluid (CSF) and blood are very useful sources of information to understand the molecular basis of the disease and also to validate some biomarkers [78]. Brain banks provide available specimens to be used in research on a wide range of neurological disorders, since most neurodegenerative diseases are mainly observed in humans [79-81]. Pioneering and subsequent studies support the importance of molecular and biochemical studies of the brain in human neurodegenerative diseases. Using the classification of LB pathology, it was observed that there is a correlation between alterations non-motor and neuropathological substrates. Stage 1 is characterized by the presence of LBs and neurites in the dorsal IX/X motor nuclei and/or intermediate reticular zone, with myentheric plexus involvement. Stage 2 comprises Stage 1 and affects also the medulla oblongata and pontine tegmentum, plus lesions in the caudal raphe nuclei, gigantocellular reticular nucleus and ceruleus–subceruleus complex, with involvement of the olfactory bulb. Stage 3 comprises Stage 2 plus lesions in the substantia nigra pars compacta. Stage 4 is characterized by basal prosencephalon and mesocortex pathology and lesions in the midbrain, pons and medulla oblongata. In Stage 5 lesions extends to the neocortex, and in Stage 6 sensory and pre-motor areas of the neocortex are also affected. Cognitive impairment and dementia barely correlate with LB pathology in the cerebral cortex [82], suggesting that other factors than mutations in α-synuclein, play key roles in PD. Biochemical alterations were already observed at very early stages of the disease. These findings suggest that,

besides morphological studies, biochemical approaches are necessary for the understanding of PD at the molecular level. For this purpose, human samples obtained from brain banks have been used in DNA studies to evaluate methylation patterns, mRNA expression, post-translational modifications, lipid composition and also to study micro RNAs and metabolomes. It is important to note that autopsy specimens should be adequately preserved for avoiding artifacts and guarantee high quality of samples. In order to reach the optimal conditions of samples, autopsies with a short post-mortem delay and the use of fresh dissection procedures in combination with optimized standard operating procedures are necessary [83].

8.9. PHARMACOLOGICAL INTERVENTION

Degeneration of nigrostriatal DA neurons results in a reduction in dopamine release and, consequently, changes in motor function. Alterations in dopaminergic stimulation alter cholinergic and glutamatergic stimulations and the symptoms become more evident. Thus, several types of pharmacological interventions were developed. [2, 5, 6, 84].

Dopamine agonists can also be used as a monotherapy in early-stage disease, or in combination with other drugs at later stages. Dopamine agonists act on postsynaptic dopamine receptors, a class of metabotropic G protein-coupled receptors that can be divided in two main groups, D1 and D2. The D1-type receptors act by Gs protein and activate adenylate cyclase, increasing cAMP synthesis, while D2 receptors reduce their activity *via* Gi protein. Nausea, dizziness, swollen ankles, confusion, hallucinations and psychosis are the main adverse effects of dopamine agonists. These adverse effects can be minimized with low initial doses and gradual dose adjustment [4].

Amantadine is a NMDA (*N*-methyl-D-aspartate) type glutamate receptor antagonist that increases dopaminergic transmission and also has a slight antimuscarinic activity. Treatment with amantadine provides a minor improvement in PD typical bradykinesia, tremor and rigidity. It is mainly used as an anti-dyskinesic agent in patients in advanced stages of PD [4].

Monoamine oxidases (MAO) are a family of enzymes that catalyze monoamine oxidation. Two types of MAO are found in humans, MAO-A and MAO-B. MAO-

B selective inhibitors (IMAO-B) lazabemide, pargyline, selegiline and rasagiline act by preventing the breakdown of monoamine neurotransmitters, such as dopamine, increasing their availability in synaptic cleft. This inhibition is irreversible, and the reuptake process is dependent on *de novo* protein synthesis. Therefore, MAO-B inhibitors can be used as a therapy to postpone the beginning of L-DOPA therapy, or as adjuvants to mitigate the effects of dopamine peaks generated by L-DOPA treatment. The rasagiline MAO-B inhibitor is a second generation drug, five times more potent than seligiline [4].

Catechol-O-methyltransferase (COMT) is an enzyme that makes the inactivation of catecholamines, such as dopamine, by the addition of a methyl group. The catecholamine L-DOPA, is a substrate of COMT. The main inhibitors of COMT (ICOMT), entacapone, stalevo and tolcapone, protect L-DOPA against COMT, prolonging the half-life of L-DOPA and, consequently, its action [85]. Generally, they are reversible inhibitors, decreasing the metabolic loss of dopamine and also increasing the half-life of L-DOPA by 30 to 50%. ICOMT are recommended for PD patients treated with L-DOPA in end-dose [4].

Anticholinergic drugs act by blocking the binding of acetylcholine to its receptors and inhibiting parasympathetic nerve impulses. Anticholinergics are divided in antimuscarinic, which comprises most of the anticholinergic drugs, and antinicotinic, which acts as muscle relaxing in most cases. Examples of anticholinergic drugs are dicycloverine, atropine, benztropine, tiotropium. These drugs reduce tremors and rigidity, but have little effect on bradykinesias. It can be also used in parkinsonism induced by antipsychotic drugs.

8.10. DIAGNOSIS

Disease progression and severity vary from one patient to another [2]. There is still no reliable diagnostic test for PD. Although neurologists generally agree that PD diagnosis requires a combination of cardinal motor signs (resting tremor, bradykinesia, postural changes), a definitive clinical classification standard has not been obtained yet.

Several studies have shown the difficulty to clinically distinguish PD from other parkinsonian syndromes. Evaluating brain autopsies of 100 patients clinically

diagnosed as having PD, the histopathology was positive for only 75% of the cases [86]. On the other hand, neurologists specialized in movement disorders reviewed the clinical and pathological diagnoses of 143 PD cases. The positive predictive histopathological analysis value of the clinical diagnosis increased to 98% [87].

Table 1: Summary of UK PDS Brain Bank Criteria for PD diagnosis

Step 1: Diagnosis of a parkinsonian syndrome	Bradykinesia plus: • muscular rigidity • rest tremor • postural instability unrelated to other primary disease.
Step 2: Exclusion criteria for PD	History of: • repeated strokes with stepwise progression • repeated head injury • antipsychotic or dopamine-depleting drugs • definite encephalitis • more than one affected relative • sustained remission • negative response to L-DOPA • unilateral features after 3 years • other neurological features • exposure to neurotoxin • presence of cerebral tumor or communicating hydrocephalus on neuroimaging.
Step 3: Supportive criteria for PD	Three or more required: • unilateral onset • excellent response to L-DOPA • rest tremor present • severe L-DOPA-induced chorea • progressive disorder • L-DOPA response for over 5 years • persistent asymmetry • clinical course of over 10 years.

Currently, the criteria of Brain Bank Society UK Parkinson's is the most widely used for diagnosis [87]. Based on this database, patients will be diagnosed with PD if they present slowness of movement (bradykinesia), one of the criteria in step 1, and at least three criteria of step 3, as described below (Table **1**).

8.11. IMAGING DIAGNOSIS

8.11.1. Single Photon Emission Computed Tomography

In single photon emission computed tomography (SPECT), gamma ray isotope labeled molecules are given to the patient by intravenous injection. Labeled cocaine derivatives, like ^{123}I-β-CIT and ^{123}I-FP-CIT (N-ω-fluoropropyl-2β-carboxymethoxy-3β-(4-iodophenyl) tropane) have been most often used, although only the latter is licensed in the UK. These markers reveal presynaptic dopamine, re-absorption site and, therefore, the presynaptic neuron can be visualized in two-dimensional images. In normal people, with neuroepileptic-induced essential tremor or psychogenic parkinsonism, the absorption is normal, but is reduced in patients with PD, PD with dementia, multiple system atrophy (MSA) or progressive supranuclear palsy (PSP). Considerable evidence supports the use of ^{123}I-FP-CIT SPECT in people with upper limb postural action tremor and to distinguish essential tremor from a dopaminergic deficiency state. ^{123}I-FP-CIT SPECT does not have high accuracy in distinguishing PD from other dopamine deficiency states, such as MSA and PSP. Future studies may demonstrate the value of this technique in the differentiation of neuroleptic medication-induced parkinsonism and psychogenic parkinsonism from a dopamine deficiency state.

8.11.2. Positron Emission Tomography

In positron emission tomography (PET), a positron emitting isotope labels a marker molecule, which is then administered by intravenous injection. The most commonly used positron emitter is ^{18}F, which can be linked to dopa or deoxyglucose. The ^{18}F-fluorodopa is absorbed by the presynaptic DA neurons of the caudate nucleus and putamen (striatum). ^{18}F-fluorodeoxyglucose (FDG) is trapped in the target tissues and is absorbed by all metabolic active and phosphorylated cells. PET has better spatial resolution than SPECT and, therefore, has a great value for differential diagnosis. However, the use of PET and FDG for distinguish PD from other parkinsonism conditions still requires additional tests for confirmation. Also, the ability of PET to differentiate PD from essential tremor was not well reported. PET is still a high cost and low-availability technique.

8.11.3. Magnetic Resonance Imaging

Structural magnetic resonance imaging (MRI) uses high intensity magnetic field to excite the hydrogen atoms present in water molecules and offer two or three-

dimensional images of the structures. This technique has been used to examine structures that are known to be involved in PD, to get valuable information for the differential diagnosis.

8.11.4. Magnetic Resonance Volumetry

Magnetic resonance volumetry uses the same structural principles of MRI to measure the size of three-dimensional volumes of structures, such as substantia nigra and basal ganglia nuclei, which are direct affected in PD. Magnetic resonance volumetry is capable of detecting volume changes in these structures and make a more precise evaluation of the severity of the lesion in PD patients possible [88].

8.11.5. Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is able to capture signals from distinct chemical nuclei inside the body. The most common detected nuclei are H, P, C Na and F. MRS is capable to detect diverse metabolites, such as cholines in cell membranes, creatine, a compound involved in energy metabolism, inositol, glucose and N-acetyl-aspartate, which is associated to the myelin sheathing [89]. Currently, MRS is mainly used as a tool for medical research, but it has the potential to provide very useful information in clinics and to be helpful in the diagnosis of PD and other neurodegenerative diseases [90].

8.12. OTHER DIAGNOSIS METHODS

8.12.1. Acute L-DOPA and Apomorphine Challenge Tests

Many PD patients show a good response to single doses of oral L-DOPA and/or subcutaneous administration of apomorphine. Acute administration of L-DOPA and apomorphine are not useful in the differential diagnosis of PD patients from those with other types of parkinsonism. Additionally, when used in the early stages of the disease, a common situation in clinical practice, acute treatment with L-DOPA and apomorphine does not distinguish PD patients from others. Moreover, acute apomorphine injection is used to assess whether advanced PD patients will still respond to dopaminergic medication [4].

8.12.2. Objective Smell Testing

Olfactory dysfunction is one of the first non-motor symptoms of PD[91]. Around 80% of people with PD may have an impaired sense of smell (hyposomia) [92].

Since olfactory loss occurs to a lesser extent or is absent in other neurodegenerative disorders, such as MSA, corticobasal degeneration and PSP, olfactory testing may be useful in differential diagnosis for PD [91].

Neurodegenerative diseases, such as PD, must have an effective early diagnosis so that treatment can be more effective. However, the current methods for diagnosis of PD are not as accurate at the onset of the disease. As a result of the low accuracy in diagnosis, PD patients are diagnosed only when symptoms are clinically detectable, and patients have already lost 50% to 70% of DA neurons [93].

New methods have been developed for premature diagnosis in groups of people who have potential risk of developing PD. Quantification of extracellular α-synuclein in cerebrospinal fluid has been proposed as a biomarker for pre-symptomatic PD [94, 95]. The production of aptamers to detect active serine protease peptidase 6 (KLK6), commonly found in neurodegenerative diseases such as PD, should be helpful for the development of new diagnostic and therapeutic strategies [96].

Current studies on molecular aspects of PD, like genetic mutations and environmental factors, together with the development of new drugs, techniques and tests to improve diagnosis accuracy, will bring new perspectives for PD therapies.

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CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

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CHAPTER 9

Huntington's Disease: A Puzzle from Childhood to Senescence

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Abstract: Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion of the huntingtin protein (htt). The progressive neuronal cell loss that takes place in the caudate-putamen and neocortical regions of HD patients leads to motor, cognitive, and psychiatric function deterioration, as well as inevitable death. Although the mutated htt is pointed as the cause of HD, it is still unknown how this mutation can promote neurodegeneration. Postmortem analyses of HD patient's brains demonstrate the presence of intracellular inclusions containing htt aggregates, which was associated to neuronal death. However, other studies suggest that inclusion formation can be neuroprotective by decreasing the levels of toxic soluble mutant htt. Moreover, many neurotransmitter systems, such as the glutamatergic, dopaminergic, endocannabinoid and trophic factor systems, are also involved in HD progression. For example, it has been demonstrated that the glutamatergic system plays an important role in the excitotoxic neuronal cell loss that takes place in HD. Despite the fact that it is clear that the main cause of HD symptoms is neuronal cell death, no therapeutic approach has yet been developed to rescue or avoid neurodegeneration. To solve this issue, a number of studies are now focusing on developing drugs that could prevent neuronal death, whereas others attempt to implement stem cells to rescue lost neurons. Both approaches have the potential to develop a disease modifying therapeutic strategy, bringing hope to HD patients.

Keywords: Apoptosis, BDNF, Ca^{2+}, caspases, cell death, chorea, dopamine, dopamine receptors, endocannabinoids, glutamate, huntingtin, Huntington's disease, mGluR5, mouse model, neurotransmission, NMDAR, protein aggregates, striatum, Δ^9-THC, trophic factors.

INTRODUCTION

George Huntington, in 1872, was responsible for the first description of Huntington's disease (HD), which was recognized as a hereditary disease

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underlying severe neurological symptoms [1]. Nowadays, it is well established that HD is an autosomal dominant neurodegenerative disorder characterized by progressive neuronal cell loss in the caudate-putamen and neocortical regions of the brain, leading to deterioration of motor, cognitive, and psychiatric functions, and inevitably leading to death [2, 3]. Cognitive and personality alterations are early symptoms, followed by chorea and loss of balance. HD patients may experience mood swings and depression in the beginning, but these symptoms may lessen as the disease progresses. However, affective disorders can be very common among HD patients, with documented rates of major depression as high as 50% [4] and mania or hypomania as high as 12% [4, 5]. HD early signs of cognitive impairment include having difficulties driving, learning new facts, and remembering belongings. As the disease progresses, accomplishing intellectual tasks becomes increasingly difficult. Chorea is the most characteristic symptom of HD, causing difficulties walking and increasing the likelihood of falls. Movement difficulties are associated with both involuntary and voluntary movement, progressively worsening over time. The most common causes of death are infections, such as pneumonia, and injuries related to falls.

9.1. HUNTINGTON´S DISEASE AND THE HUNTINGTIN PROTEIN

HD is caused by a polyglutamine expansion in the amino-terminal region of the huntingtin (htt) protein [6]. In the human genome, the htt gene is present in the chromosome 4 and the exon-1 of htt normally contains between 6 and 35 CAG repeats, whereas HD patients exhibit 36 or more CAG repeats [6]. Importantly the length of the polyglutamine repeat inversely correlates with the age of disease onset and directly correlates with the severity of symptoms [7]. However, patient sex, environmental factors and genetic modifiers can alter HD onset and progression.

Although htt mutation has been discovered more than 20 years ago, the mechanisms responsible for mutant htt pathogenicity are still largely unknown. So far, it is still unclear why the mutant protein, which is expressed throughout the body, promotes selective loss of striatal medium sized spiny neurons (MSNs). Moreover, it is still not clear whether a lack of function of the htt protein or a gain of toxic function of the mutant htt plays the most important role in HD pathology. Htt knockout in mice leads to embryonic lethality as a result of increased

apoptosis and heterozygous mice exhibit severe cognitive deficits due to morphological changes in the subthalamic nucleus of the basal ganglia [8, 9]. Htt antiapoptotic effect is likely due to both inhibition of caspase-3 activity and activation of prosurvival pathways involving the kinase Akt [10, 11]. On the other hand, mutant htt triggers a cascade that leads to neuronal dysfunction through oxidative stress, transcriptional dysregulation, glutamate excitotoxicity, activation of apoptotic cascade, mitochondrial dysfunction, and energy depletion [12-15].

Similarly to what is observed in other neurodegenerative diseases such as Alzheimer´s disease (AD), HD is characterized by protein aggregates that accumulate within cells. Immunohistochemical analyses of postmortem brain tissue of HD patients demonstrate the presence of intracellular inclusions containing htt aggregates, which are associated with the selective loss of striatal MSNs [16, 17]. HD MSNs present in the striatum, containing GABA and enkephalin, are affected early in the disease and are the primary neurons targeted in HD. Over time, htt aggregates and inclusions spread to the remainder of the basal ganglia with subsequent dissemination through the cortex and substantia nigra. Importantly, htt aggregate formation and neuronal loss strongly correlate with HD symptom severity [18].

Aggregates are formed mostly from the amino-terminal fragments containing the polyglutamine repeats, which are cleaved of polyglutamine expanded htt and accumulate in neurites, cytoplasm, and nuclei. Considerable evidence indicates that htt cleavage by caspases is important in HD pathogenesis [19]. A transgenic mouse model expressing mutant htt identical to that expressed by YAC128 mice, except for a point mutation rendering mutant htt resistant to caspase-6 cleavage, is resistant to the excitotoxicity, neurodegeneration, and behavioral characteristics of YAC128 mice [20, 21]. These data strongly indicate that mutant htt proteolysis is required for the formation of toxic htt fragments.

Although a number of studies indicate that htt aggregates contribute to synaptic damage in HD, it has been suggested that inclusion formation protects neurons from cell death, possibly by decreasing the levels of toxic soluble forms of mutant htt [22, 23]. Supporting this hypothesis, a recent report shows that synaptic activity dependent on N-methyl-D-aspartate glutamate receptor (NMDAR)

increases htt inclusion formation and diminishes mutant htt toxicity [24]. In contrast, activation of extrasynaptic NMDARs increases the vulnerability of neurons expressing mutant htt to cell death and activates cell signaling pathways that can disaggregate mutant htt [24]. Thus, it is not clear whether htt aggregation contributes to neuronal cell death or promotes neuroprotection.

9.2. NEURONAL CELL DEATH IN HUNTINGTON´S DISEASE

HD symptoms are caused by the neuronal cell loss that takes place in the striatum and neocortical regions of the brain. The striatum, comprised of the caudate nucleus and putamen, represents the major "input" stage of the basal ganglia, being mainly composed of projection neurons (up to 95%) and a much smaller number of interneurons (approximately 5%). Striatal projection neurons are all GABAergic and morphologically characterized by a long axon, medium-sized cell bodies, and spiny dendrites, hence the commonly used term of medium sized spiny neurons (MSNs) [25, 26]. MSNs represent the main and earliest striatal cell type affected in HD, whereas striatal interneurons are typically unaffected or only mildly affected at late stages of the disease. Although less affected than striatal neurons, cortical neurons might also undergo cell death due to htt mutation. Interestingly, in the cortex, as in the striatum, large pyramidal projection neurons are preferentially lost and small interneurons are preserved in HD [27, 28]. Htt is widely expressed in the brain and in non-neuronal tissues and not particularly enriched in the striatum [29, 30]. Thus, it is still unknown why MSNs are particularly affected in HD.

Although htt mutation is well established as the cause of HD, it is still unclear how the mutated htt protein promotes neuronal cell death (Fig. **1**). Recent publications indicate that htt aggregate might not be the major cause of neuronal death, but that mutated htt toxic effects could play an important role in neurotoxic processes [16, 17, 22, 23]. Mutated htt can alter gene transcription, induce apoptosis, and disrupt key neuronal functions such as proteasomal function, ubiquitination, axonal transport, endocytosis, and synaptic transmission [12-15]. Moreover, polyglutamine expanded htt can increase intracellular Ca^{2+} levels, which may contribute to the neuronal cell death that takes place in HD. NMDAR sensitization can be induced by mutant htt protein, increasing Ca^{2+} influx into the

cell [31, 32]. In addition, mutant htt protein can also cause an increase in intracellular Ca^{2+} levels by promoting mitochondrial Ca^{2+} regulation destabilization [33, 34] and by leading to sensitization of InsP3 receptor-mediated release of Ca^{2+} from intracellular stores [35, 36]. The result of InsP3 receptor sensitization is the increased stimulation of $G\alpha_{q/11}$ coupled receptors, leading to an increase in Ca^{2+} release from intracellular stores [36, 37].

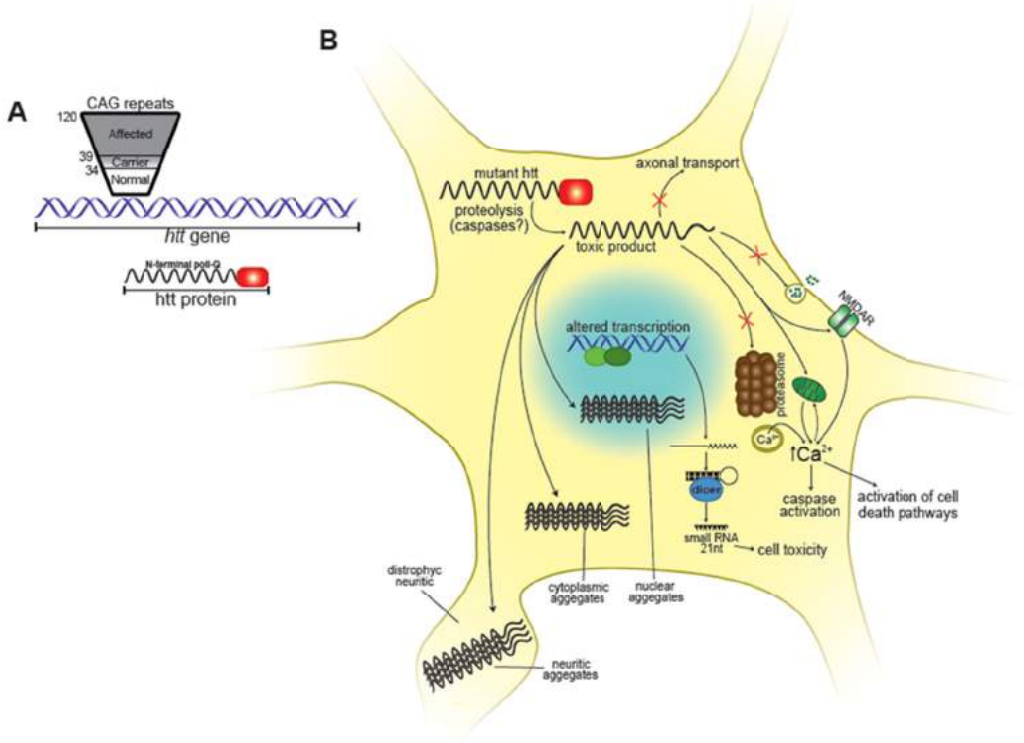

Figure 1: Toxic pathways activated by mutant htt. Mutant htt protein can promote toxicity and neuronal cell death through a number of mechanisms. The long amino-terminal poly-glutamine expansion of the mutated form of the htt protein is cleaved by caspases, which appears to be important for the mutant htt-mediated toxic effects. Mutated htt can alter gene transcription, induce apoptosis, and disrupt key neuronal functions such as proteasomal function, ubiquitination, axonal transport, endocytosis, and synaptic transmission. Mutant htt leads to N-methyl-D-aspartate receptor (NMDAR) sensitization, increasing Ca^{2+} influx into the cell, which may contribute to neuronal cell death. The htt cleaved product is also capable of triggering a cascade that leads to mitochondrial dysfunction, increasing the intracellular Ca^{2+} levels and disturbing cell metabolism. Moreover, mutated htt can also sensitize InsP3 (IP_3), leading to increased levels of intracellular Ca^{2+}. Furthermore, transcripts containing repeats can form hairpin structures that are substrate for the ribonuclease dicer, generating short RNA duplexes and activating RNA interference (RNAi) responses, resulting in cell toxicity.

Mutant huntingtin protein promotes mitochondrial Ca^{2+} abnormalities early in HD pathogenesis in a direct manner, as incubation of mitochondria from normal human lymphoblast with a polyglutamine-expanded protein fragment recapitulates the mitochondrial Ca^{2+} defect observed in HD [33]. It has also been shown that the huntingtin protein binds to the outer membrane of human immortalized cells mitochondria and of cultured striatal cells mitochondria from WT and transgenic mice [34]. In addition, binding of mutated huntingtin protein, but not of wild type, enhances sensitivity to Ca^{2+}-induced opening of the MPT, promoting the release of cytochrome c from mitochondria obtained from normal liver [34]. Mitochondria is important for buffering cytoplasmic Ca^{2+} and increased neuronal Ca^{2+} can modify mitochondrial ATP production by uncoupling oxidative phosphorylation [38]. Increased cytoplasmic Ca^{2+} may promote discharge of the mitochondrial membrane potential, opening of the mitochondrial permeability transition (MPT) pore, release of cytochrome c, and activation of cell death pathways [38]. Mitochondrial dysfunction resulting from Ca^{2+} overload, prolonged membrane depolarization or impaired electron transfer chain is the main source of intracellular reactive oxidative species [39, 40]. Interestingly, a link between NMDAR stimulation and mitochondria deregulation has been established, as the reduced mitochondrial ATP levels and decreased ATP/ADP ratio found in mutant htt-containing striatal cells is normalized by blocking NMDA receptor-mediated Ca^{2+} influx [41].

Recently published data indicate that, in addition to the deleterious effect generated by the mutated htt protein, the mutant CAG htt repeats are toxic at the RNA level [42]. Transcripts containing long CUG and CAG repeats form hairpins structures that are substrate for the ribonuclease dicer, generating short RNA duplexes and activating RNA interference (RNAi) response [43]. The 21 nucleotide long fragments generated by dicer act as endogenous siRNAs and trigger downstream silencing effects [43]. The expanded htt exon-1 mRNA containing 40 or more CAG repeats induces cell death and increases levels of 21 nucleotide long CAG-repeated small RNAs [42]. Importantly, the higher the number of CAG repeats, the greater is cell toxicity [42].

9.3. HUNTINGTON'S DISEASE AND THE GLUTAMATERGIC SYSTEM

Glutamate, the major excitatory neurotransmitter in the brain, is essential for a wide variety of physiological processes, such as memory, cognition and neuronal

cell development. Nevertheless, glutamate has also been implicated in the pathogenesis and neuronal cell loss that takes place in HD [44-47]. Glutamate exerts its actions by activating ionotropic glutamate receptors, which are ligand-gated ion channels that mediate fast excitatory neurotransmission, and metabotropic glutamate receptors (mGluRs), which are members of the G protein-coupled receptor (GPCR) family [48-52]. Three different types of ionotropic glutamate receptors have been identified, NMDA, AMPA, and kainate receptors, and eight distinct mGluRs, which are divided into three subgroups based on sequence homology and G protein coupling-specificity [47, 48, 53, 54]. Group I mGluRs (mGluR1 and mGluR5) promote activation of phospholipase C (PLC) *via* $G\alpha_{q/11}$, whereas Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs inhibit adenylyl cyclase *via* $G\alpha_{i}$.

Excitotoxicity is a very well-known process of neuronal cell death, and also plays an important role in many CNS disorders, including ischemia, trauma, and neurodegenerative disorders, such as AD, HD, and Parkinson's disease (PD) [55, 56]. Excitotoxicity occurs as a result of increased release of extracellular glutamate or a reduction in its removal from the synaptic cleft, which causes glutamate receptor over-stimulation and increased Ca^{2+} levels, as well as mitochondria dysfunction and cell death [57, 58]. Increased Ca^{2+} levels induced by glutamate stimulation is mainly achieved by activation of NMDAR and, to a lesser degree, by Group I mGluRs, which are coupled to Ca^{2+} release from intracellular stores [50, 54].

It has been shown that NMDARs play a role in the excitotoxic neuronal cell loss that occurs in HD. *Post-mortem* brain tissue from HD patients in the early symptomatic phase exhibit loss of striatal NMDARs, suggesting that striatal neurons expressing high levels of NMDAR are more susceptible and are lost early during disease progression [59, 60]. The role of NMDAR in HD can be further highlighted by the fact that a mouse model of HD was generated by injecting the NMDAR agonist quinolinic acid into the striatum [61, 62]. This HD mouse model recapitulates many HD features, including HD-like lesions and symptoms. Furthermore, the reason why MSNs are more susceptible in HD appears to be dependent on the type of NMDARs that these neurons express. Mutant htt protein is capable of sensitizing NMDARs that are comprised of the NR1A/NR2B, but

not NR1A/NR2A [31, 63]. Interestingly, although other brain structures express combinations of both NR2A and NR2B with a variety of NR1 splice variants, MSNs essentially express the NR1A and NR2B subunits [64-66]. Thus, the type of NMDAR subunit expressed might underlie the preferential death of MSNs in the striatum.

Recent published data indicate that synaptic NMDAR activity drives neuroprotective gene transcription, promoting neuronal resistance to mutant htt-mediated cell death [24]. In contrast, stimulation of extrasynaptic NMDARs increases the vulnerability of neurons expressing mutant htt to cell death [24]. In agreement with these findings, it has also been demonstrated that extrasynaptic NMDAR expression and current are increased in the striatum of an HD mouse [67]. Moreover, nuclear CREB activation was reduced in HD mouse striatum [67]. Thus, the balance between synaptic and extrasynaptic NMDAR activity may be crucial to determine neuronal cell survival in Huntington's disease. In support to this hypothesis, low concentrations of the NMDAR antagonist, memantine, which maintain physiological synaptic activity while blocking excessive extrasynaptic NMDAR stimulation, lessen mutated htt-mediated excitotoxicity [24, 67].

It is not yet clear whether Group I mGluRs have a role in HD. However, a direct link between htt and Group I mGluRs has been established, as mGluR1/5 interacts with both htt and optineurin, which is also an htt-interacting protein [68, 69]. Nevertheless, the role of mGluR5 in HD-mediated neuronal cell death is very controversial. Treatment of an HD transgenic mouse model with an mGluR5 antagonist increases survival [70]. In addition, disturbed Ca^{2+} signaling and apoptosis observed in primary cultured striatal neurons from an HD mouse model has been attributed to activation of mGluR1/5 and NMDAR containing the NR2B subunit [35, 36]. Nevertheless, data from other groups have provided evidence that Group I mGluRs activation can be protective. For example, NMDA-mediated excitotoxicity can be attenuated when cortical neuronal cultures are consecutively incubated two times with the group I mGluRs agonist, DHPG (3,5-dihydroxyphenylglycine) [71]. In rat hippocampal slices, mGluR1 activation by DHPG protects CA1 hippocampal cells [72]. In addition, it has been demonstrated that mGluR1/5 signaling is modified in a mouse model of HD during the presymptomatic phase of the disease and that these alterations have a protective role

[69, 73]. Activation of mGluR1/5 expressed in striatal neurons from a mouse model of HD leads to high levels of Ca^{2+} release from intracellular compartments, which can contribute to excitotoxic processes (Fig. **2**) [35, 73]. However, mGluR1/5 stimulation also leads to activation of other signaling pathways important for cell survival, such as extracellular signal-regulated kinase (ERK) and AKT [74-76]. Interestingly, mGluR5 activation leads to higher levels of ERK and AKT activation in HD than in control neurons (Fig. **2**) [73]. In addition to its known neuroprotective role, Akt can promote phosphorylation of mutant htt protein, which leads to reduced htt aggregate formation and neuronal cell death, providing an extra protective pathway in HD [10, 77]. Thus, depending on the context of activation, group I mGluR stimulation is found to be either neurotoxic or neuroprotective.

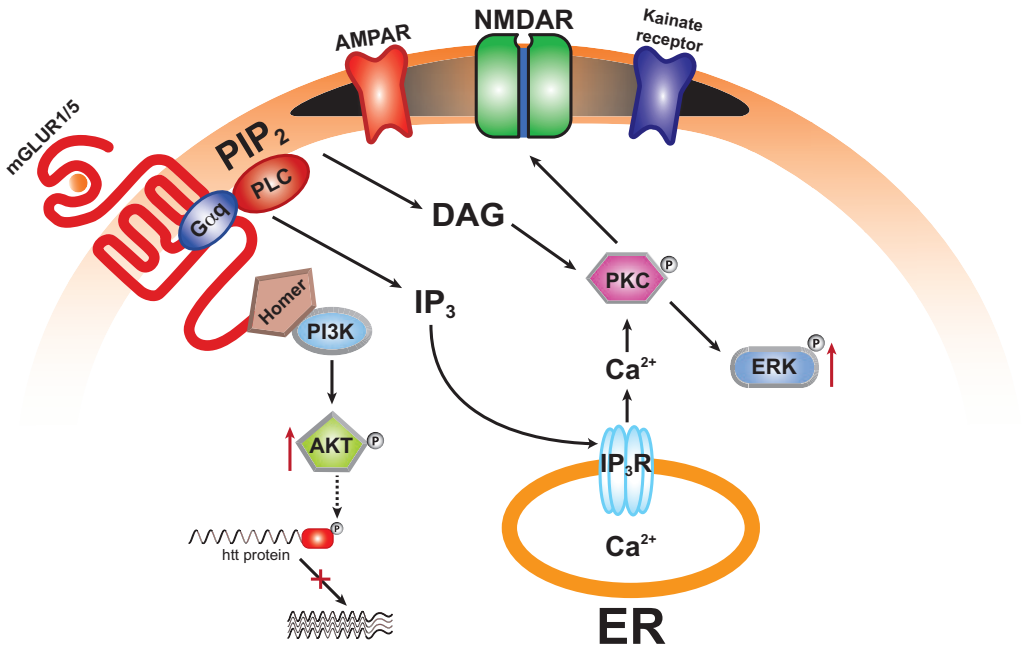

Figure 2: Cell signaling pathways activation by Group I mGluRs. Group I mGluRs activate the hydrolysis of PIP_2 by phospholipase C (PLC) following the activation of the heterotrimeric G protein Gαq resulting in increases of intracellular diacylglycerol (DAG) and InsP3 (IP_3) levels. InsP3 activates the InsP3 receptor (IP_3R) resulting in increased intracellular Ca^{2+} concentrations which in conjunction with DAG activate protein kinase C (PKC). PKC activation can lead to the activation of ERK1/2 phosphorylation and the phosphorylation of the NMDA receptor. Homer interacts with the carboxyl-tail of Group I mGluRs and can either contribute to the activation of Akt *via* PI_3 kinase (PI_3K) or can directly regulate NMDA receptor activity *via* its association with Shank. Adapted from: Ribeiro et. al, Molecular Neurobiology, 2011, 43:1-11.

9.4. HUNTINGTON'S DISEASE AND THE DOPAMINERGIC SYSTEM

Striatal MSNs receive glutamatergic input from the cortex and dopaminergic input from the substantia nigra [78]. Additionally, a number of reports indicate that dopamine might play a role in HD-mediated neuronal death and chorea symptoms [79]. In agreement with the hypothesis that dopamine plays a role in HD, tetrabenazine, which is a dopamine antagonist with anti-chorea properties, is the only FDA approved drug to treat HD patients [80]. Dopamine receptors are highly expressed in MSNs and the expression levels of D1 and D2 dopamine receptors are reduced in HD basal ganglia even before any neuronal cell death can be observed [81, 82]. Moreover, mouse models of HD show a corresponding loss of D2 dopamine receptor expression early in the progression of neuronal pathology [83, 84]. Thus, dopamine receptors are markers of early HD-related alterations.

Increased levels of extracellular dopamine observed in knockout mice for the dopamine transporter leads to both spontaneous striatal death and behavioral alterations that resemble HD [85]. Moreover, exacerbation of HD symptoms and augmentation of neuropil aggregate formation occur when these mice are mated to a knock-in HD mouse model [86]. Stimulation of primary cultured striatal neurons with dopamine leads to the activation of pro-apoptotic pathways, formation of htt aggregates, and disturbance of mitochondria *via* D2 dopamine receptor activation [87, 88]. In addition to increase aggregate formation, dopamine also intensifies mutant htt toxicity in striatal neurons through the production of reactive oxygen species (ROS), which activates the pro-apoptotic c-Jun amino-terminal kinase (JNK)/c-Jun pathway [87]. Highlighting the importance of D2 receptor in HD, it has been demonstrated that haloperidol, which is a D2 receptor antagonist, protects striatal neurons from mutated htt toxicity [89]. Moreover, D2 receptor knock-down using siRNA abrogates the deleterious effects produced by dopamine [90]. Aggregate formation prompted by D2 activation involves a Rho/ROCK signaling pathway, as ROCK activity inhibition reverses D2-mediated aggregate formation, neuritic retraction and neuronal cell death induced by mutant htt [90].

Dopamine and glutamate can also synergistically induce apoptosis of MSNs *via* increased Ca^{2+} signaling, as stimulation of MSNs from a transgenic mouse model of HD with both glutamate and dopamine leads to high levels of cell death [91,

92]. Moreover, it has been shown that mutant htt increases the sensitivity of striatal cells to dopamine and glutamate inputs by altering a common NMDAR and D1 dopamine receptor downstream pathway such is Cyclin-dependent kinase 5 (Cdk5) [92]. Cdk5 activation by p35 is essential for brain development [93]. However, Cdk5 becomes a cell death inductor when it binds to p25, the calpain-mediated cleaved product of p35 [94]. Increased intracellular Ca^{2+} promoted by stimulation of both NMDA and dopamine receptors leads to higher calpain activity that results in enhanced cleavage of p35 into p25 [94]. As mutated htt protein leads to high levels of intracellular Ca^{2+} by sensitizing NMDARs and InsP3Rs, p25 can increase in HD to levels that will exacerbate neuronal cell death processes [92].

9.5. HUNTINGTON'S DISEASE AND THE CANNABINOID SYSTEM

Endocannabinoids (ECs), as well as Δ^9-tetrahydrocanabinol (Δ^9-THC), can bind and activate two cannabinoid receptors named CB1 and CB2 [95, 96]. Five ECs have been identified thus far, including anandamide (AEA) and 2- arachidonoyl glycerol (2-AG), which are the two most studied ECs [97, 98]. ECs acts as retrograde messengers at many synapses in the central nervous system, which means that ECs are released by postsynaptic neurons and act predominantly at presynaptic neurons [99]. This retrograde signaling pathway has emerged as being important in synaptic plasticity and in numerous neurophysiological functions such as pain, appetite, learning and memory, and motor functions [100-103]. Moreover, as brain regions involved in cognition and motor activity express high levels of CB1 receptors, it has been suggested that ECs play a role in a variety of CNS disorders, especially neurodegenerative diseases [104].

Within the basal ganglia, the main function of the cannabinoid system is to modulate GABAergic and glutamatergic synapses through a retrograde signaling mechanism [105]. Thus, activation of EC receptors has profound effects on the control of movement [106, 107]. For example, AM404, which is an anandamide transport inhibitor, reduces the stimulation of motor behaviors elicited by the selective D2 receptor agonist quinpirole [107]. The role of ECs in movement control implicates this system in movement disorders such as HD. Glass *et al*. [108] were the first to provide evidence linking EC signaling with HD, as they

demonstrated loss of about 97% of CB1 receptors in the substantia nigra of human HD brains [108]. In HD patients, deficiency of CB1 receptor levels precede the loss of D1 and D2 dopamine receptors and occur even before the onset of major HD symptoms [82]. In transgenic mouse models of HD, the decrease in CB1 receptor mRNA takes place before the onset of motor symptoms [109]. Moreover, CB1 receptor expression levels continue to decrease as disease progresses [110]. Interestingly, it has been reported in the R6/1 transgenic model of HD that environmental enrichment upregulates CB1 receptor binding and provides behavioral improvement [111]. In agreement with these data, it has also been shown that lack of CB1 receptor expression aggravates the motor performance of a transgenic mouse model of HD [112]. These data indicate that early loss of CB1 receptor may be detrimental in HD and that activation of CB1 receptor pathways could afford protection.

9.6. HUNTINGTON'S DISEASE AND TROPHIC FACTORS

Trophic factors, such as brain-derived neurotrophic factor (BDNF), play a crucial role in neuronal survival and function [113]. Wild-type, but not mutant, huntingtin can increase vesicular transport and transcription of BDNF [114, 115]. Thus, mutation of the htt protein results in decreased BDNF transcription and axonal transport, which negatively affect the survival of both striatal and cortical neurons [114, 115]. Moreover, mutated htt also decreases BDNF secretion by cortical astrocytes [116]. Wild-type htt increases BDNF transcription by sequestering the repressor element-1 transcription factor/neuron restrictive silencer factor (REST/NRSF), the transcription factor that binds to the neuron restrictive silencer element (NRSE) on BDNF promoter II [117]. Wild-type huntingtin interaction with REST/NRSF is much stronger than that of mutated htt, which might explain why less BDNF is transcribed in HD [117].

Most of BDNF neuroprotective effects are mediated by the receptor tyrosine kinase-B (TrkB)-induced activation of pro-survival signaling pathways, including: PLC-γ, Ras/MEK/MAPK and PI3K/Akt pathways [118]. In accordance with that, it has been shown that BDNF protects cortical neurons from 3-NP toxicity through the activation of PI3K and ERK1/2 intracellular signaling pathways resulting in decreased mitochondrial abnormalities and apoptosis [119]. In striatal

neurons, it has been demonstrated that BDNF alters the expression of genes involved in striatal neurite outgrowth by activating the MEK/ERK1/2 signalling pathway [120].

It has been shown that BDNF is capable of preventing the death of MSNs in a quinolinic acid model of HD and in a 3-NP-induced toxicity mouse model, which are HD mouse models that exhibit movement alterations, cognitive deficits and neuronal loss similar to those seen in HD patients [121-123]. Intrastriatal BDNF delivery and selective forebrain over expression of BDNF reverse cortical and striatal injury, and improve motor performance in transgenic HD mice [124-127]. Moreover, super-expression of BDNF by striatal astrocytes of an HD transgenic mouse model delays the onset of the HD-related motor phenotype [128]. Thus, increased levels of BDNF ameliorate HD-related symptoms.

9.7. THERAPEUTIC PERSPECTIVES IN HUNTINGTON´S DISEASE

Tetrabenazine, which is the only FDA-approved drug to treat HD, acts primarily through reversible inhibition of the vesicular monoamine transporter 2 (VMAT2), a presynaptic neuronal transporter responsible for concentrating monoamines into vesicles [129, 130]. Inhibition of VMAT2 results in presynaptic dopamine depletion, with lesser reductions in norepinephrine and serotonin [131]. Tetrabenazine reduces chorea, but has detrimental effects on cognition and depression [132]. Antipsychotics are also employed to control chorea; however, as tetrabenazine, they have no disease modifying actions [132]. Thus, the development of a therapeutic strategy to delay HD progression is a very important undertaking currently.

The conspicuous role of NMDARs in neuronal excitotoxic cell death has led to the development of NMDAR antagonists such as ketamine, amantadine, and memantine for treating neurological disorders caused by neuronal cell death. Riluzole, which is approved to treat amyotrophic lateral sclerosis, is known for reducing excitotoxicity *via* suppression of glutamatergic neurotransmission and has potential as a disease modifying drug. Animal models of HD treated with riluzole exhibited reduced striatal degeneration and improved associated motor dysfunction [133-135]. Moreover, riluzole was found to increase HD serum

concentration of BDNF, which is a factor important for striatal neuronal survival and that has its concentration reduced in HD [136, 137]. However, ketamine and riluzole have been tested in HD patients and these drugs were found to have little or no symptomatic benefit [138-140]. In a number of studies, amantadine seems to alleviate chorea, although suppression of chorea varied widely among patients [141]. Nevertheless, evidence from other double-blind crossover trials has shown insignificant results between amantadine and placebo [142, 143]. This mixed body of evidence leads to uncertainty regarding amantadine's efficacy for routine use in HD.

Neuronal culture studies demonstrate that the positive or negative consequences of NMDAR activity are determined by the subcellular location of the receptor [24, 144, 145]. According to these studies, stimulation of synaptic NMDARs results in the activation of prosurvival pathways, although stimulation of extrasynaptic NMDARs privileges cell death. Memantine, different than other NMDAR antagonists, preferentially blocks extrasynaptic NDMARs [146]. In agreement with these hypothesis, it has been shown that treatment of a transgenic mouse model of HD with low doses of memantine blocks excessive NMDAR stimulation (extrasynaptic) without affecting physiological synaptic transmission (synaptic), ameliorating neuropathological and behavioral symptoms related to HD [24, 67]. A pilot study indicates that HD patients exhibit diminished chorea following memantine 20 mg/day treatment for 3 months [147]. However, no improvements in cognitive, behavioral, and functional aspects were observed [147]. Despite the uncertain symptomatic results with memantine, its potential as a neuroprotective drug has led to further clinical trials that are currently underway to investigate memantine and its effects on cognitive and behavioral functions in HD patients with cognitive impairment.

Drugs acting on the cannabinoid system have also been tested in HD. Botanical extracts enriched in either Δ^9-THC or cannabidiol (CBD), which are the main components of the cannabis-based medicine Sativex, provide neuroprotection in rat models of HD [148, 149]. The administration of Δ^9-THC- and CBD-enriched botanical extracts ameliorates HD-mediated effects, such as down-regulation of CB1 receptor and IGF-1 expression, and up-regulation of calpain expression, whereas it completely reverses the reduction in superoxide dismutase-1

expression [148]. Interestingly, these effects were not blocked by selective antagonists of either CB1 or CB2 receptors, indicating that the protective effects are caused by the antioxidant and cannabinoid receptor-independent properties of these phytocannabinoids [148, 149]. CBD has also been studied in rats lesioned with malonate, a model of striatal atrophy. CBD alone did not provide protection in rats injured with malonate, as only CB2 receptor agonists were effective [150]. However, the combination of CBD with Δ^9-THC used in Sativex was highly effective in preserving striatal neurons in this model in a mechanism involving both CB1 and CB2 receptors [151]. CBD combined with Δ^9-THC is currently facing phase II-clinical trials.

Therapeutic approaches targeting an increase in BDNF might be a strategy to slow or prevent HD [152]. Research on BDNF and HD has focused on drugs that could boost BDNF production, as this trophic factor does not cross the blood-brain barrier. Moreover, the positive modulator of AMPA glutamate receptors, ampakine, is capable of up-regulating endogenous BDNF levels, rescuing neuronal plasticity and diminishing learning problems observed in a HD mouse model [153]. However, ampakine treatment has no positive effect on motor alterations. However, as ampakines do not pose major adverse effects, this class of drugs may represent a good option for treating the cognitive decline that occur in HD, as well as for preventing neuronal cell loss [153].

Intrastriatal injections of adenovirus encoding BDNF demonstrated neuroprotection of striatal neurons in quinolinic-acid lesioned rats [154]. Moreover, cells that express and continuously release BDNF have been engineered as a new tool to boost BDNF levels [121, 123, 155, 156]. One of these studies demonstrates that BDNF-secreting cells do not provide robust neuroprotection [155]. However, other studies have demonstrated that BDNF-secreting cells provide improvement in motor performance and reduction of striatal neurons damage [121, 123, 156]. These data highlight the importance of cell transplantation and also BDNF for the treatment of neurodegenerative diseases, such as HD.

A potential approach to restore striatal MSNs includes the use of stem cells that could be surgically transplanted into the striatum of HD patients. A number of

small pilot studies have demonstrated the feasibility of this strategy, but as the majority of studies have used human fetal tissues, ethical controversies and regulatory constraints have limited this approach [157-162]. Striatal neurons have been differentiated from human embryonic stem cells (hESCs) and it has been shown that these neurons were capable of integrating into the host neural circuitry and correct motor deficits in a rodent model of striatal degeneration [163-165]. However, although a number of studies demonstrate that cell transplantation is a potential option to treat HD, the benefits of this intervention does not last for long periods of time. A cell transplantation trial demonstrated that 3 of 5 patients exhibited motor and cognitive stabilization or improvement up to 2 years post-intervention; however, the benefits dissipated between 4 and 6 years following surgery [157, 158]. In another trial, modest improvements were realized in 6 out of 7 patients, but again lasting for only 2 years [159, 160]. Larger clinical trials are currently being conducted to further evaluate the clinical efficacy of neurotransplantation in HD [166, 167].

The recent landmark discovery that somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) creates a new strategy to treat neurodegenerative diseases [168]. A recent report demonstrates that iPSCs derived from HD patient fibroblasts can be corrected by replacing the expanded CAG repeat with a normal repeat using homologous recombination, and that correction of HD-iPSCs normalizes pathogenic HD signaling pathways and reverses disease phenotypes such as susceptibility to cell death and altered mitochondrial bioenergetics in a HD mouse model [169]. iPSCs, differently than hESCs, do not face ethical constraints as they are not produced from embryonic human cells. Moreover, iPSCs are patient specific and will not activate host immune responses [170]. Future technical developments will be important to make the use of iPSCs feasible to restore striatum loss and ameliorate HD-related symptoms in patients.

As the genetic alteration underlying HD involves one single gene, inhibiting htt expression is a promising therapeutic option. Antisense oligonucleotides that block htt expression have been shown to alleviate symptoms and prolong survival in mouse HD models [171-173]. However, as HD is dominantly inherited, patients exhibit both wild-type and mutant htt alleles. Blocking expression of both wild-

type and mutant htt might have deleterious effects [8, 9]. Thus, allele selective inhibition could afford improved therapeutic efficacy. It has recently been shown that single-stranded RNA targeting the htt CAG repeat is capable of inhibiting mutant htt allele expression selectively [174]. Future clinical trials will be important to demonstrate whether htt silencing can delay HD progression in patients. Although gene silencing appears to be an attractive and promising future therapy for HD, delivery of oligos to the central nervous system is still a concern. RNA oligos do not cross the blood brain barrier and would have to be introduced to the cerebral spinal fluid during the entire life of HD patients to be effective.

9.8. CONCLUDING REMARKS

The identification of the mutation that causes HD in 1993 was crucial to develop animal models to study the disease and to better understand HD mechanisms. HD, as other neurodegenerative diseases, is a very complex disorder involving many neurotransmitter systems and different areas of the brain. Although it is clear that the main cause of HD symptoms is the neuronal cell death that takes place in the caudate-putamen and neocortical regions of the brain, no therapeutic approach has yet been developed to rescue or avoid neuronal cell loss. A number of studies focus on developing drugs that could prevent neuronal death, whereas others attempt to implement stem cells to rescue lost neurons. Both approaches have the potential do develop a disease modifying therapeutic strategy, bringing hope to HD patients.

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CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

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CHAPTER 10

Amyotrophic Lateral Sclerosis: A Role for Non-Neuronal Cells

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Abstract: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive degeneration of both upper and lower motor neurons leading to paralysis and finally to death. Non-neuronal cells, including glial cells, have been shown to actively participate in the physiopathological process occurring in ALS. Experiments using chimeric mice expressing ALS-linked mutations suggest that neighboring non-neuronal cells modulate disease phenotype. In this review, recent findings involving the role of astrocytes, microglia and of other non-neuronal cells will be discussed. The study of motor neuron microenvironment could lead to a better understanding of the physiopathology of ALS to find new pathways to slow down motor neuron degeneration.

Keywords: Amyotrophic lateral sclerosis, ALS, amyotrophia, animal models, astrocytes, CNS, glial cells, microglia, motor neuron, motor neuron disease, neurodegeneration, neuroinflammation, neuroprotection, neurotoxicity, non-cell autonomous, non-neuronal cells, progressive paralysis, SOD1, spinal cord, transgenic mice.

10.1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS), originally described by Charcot in 1869, is the most prevalent type of adult motor neuron disease, characterized by the progressive dysfunction and degeneration of both upper motor neurons in the brain cortex and lower motor neurons located in the brainstem nuclei and ventral spinal cord. The degeneration results in progressive paralysis, muscle atrophy and death due to respiratory failure after 2-5 years of symptoms onset [1, 2]. The disease has an annual incidence of 1-2 per 100,000 and represents a major cause of acquired non-traumatic disability [3]. The average age of clinical onset of ALS is 55-60 years old, with male gender, increasing age and family history being the

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main risk factors [4-6]. The variability in clinical disease duration is large, with some patients dying within months after onset and others surviving for more than two decades [7].

In the past years several lines of investigation have demonstrated that ALS is a heterogeneous syndrome rather than a unique disease [2]. Whereas certain brain functions, including oculomotor and sphincter function, are relatively spared, ALS may be associated with non-motor symptoms, like cognitive dysfunction (20–50% of cases). For instance, frontotemporal dementia (FTD), a degenerative disorder of the frontal and anterior temporal lobes, occurs in 5–15% of ALS patients, characterized by marked executive dysfunction and behavior change [8] and these patients have a shorter survival [9].

The cause of the disease remains unknown for the majority of sporadic cases of ALS. There is currently no effective disease-modifying treatment other than Riluzole, which only has a modest effect on survival [10]. An improved understanding of the pathophysiology of ALS has potential for novel and more effective therapeutic intervention.

10.2. GENETIC CAUSES OF ALS

ALS is classified as hereditary or familial ALS (FALS) and sporadic ALS (SALS) with similar clinical characteristics. FALS accounts for 5% of patients if only first-degree relatives are taken into account; this percentage increases when distant relatives are included [11] and is mostly of autosomal dominant inheritance [reviewed in 12]. The most frequent cause of FALS are the recently described intronic GGGGCC repeat expansions in the chromosome 9 open reading frame 72 (C9ORF72) [13, 14]. C9ORF72 repeat expansions were found to be linked both to ALS and frontotemporal dementia and accounted for around 40% of patients with familial ALS worldwide and 6% of patients with sporadic ALS [15, 16].

Mutations in the gene encoding the antioxidant enzyme Cu/Zn superoxide dismutase-1 (SOD1) are the second most frequent cause of familial forms of ALS (15%) [17]. SOD1 is an enzyme composed of 153 amino acids and is involved in

free radical scavenging, in which more than 150 different mutations (mostly missense mutations) have been reported to be pathogenic [12]. Toxicity of mutant SOD-1 involves a dominant gain-of-function rather than a merely diminished superoxide-scavenging activity [1, 18, 19]. This is supported by findings showing that deletion of mouse endogenous SOD1 gene did not lead to an ALS phenotype [20] and patients carrying either an active or inactive form of the enzyme both develop ALS. Transgenic mice and rats expressing SOD1 [21-26] recapitulate many features of ALS pathology and represent the main model for studying this disease at present. Importantly, the expression of the human wild-type SOD1 at similar levels [21, 23] did not lead to ALS phenotypes. Several disease mechanisms have been proposed in the SOD1 animal models of the disease that include: protein aggregation, oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, axonopathy, endosomal trafficking and neuroinflammatory processes [1, 27-33].

Other genetic causes of FALS include mutations in TARDBP [34-38]. This gene encodes for TAR DNA-binding protein 43 (TDP43), a protein present in ubiquitin-positive, tau-negative, neuronal inclusions in most patients with ALS or FTD [39]. A wide number of the mutations are missense mutations, located in exon 6 of TARDBP, which encodes the C-terminal glycine-rich part of the protein; however few mutations are deletions that give rise to a protein truncated at the C-terminal [12, 40]. Remarkably, TARDBP mutations can lead to ALS with or without FTD but only rarely to FTD alone [12]. TDP43, belongs to a family of heterogenous ribonucleoproteins that bind DNA and RNA and is normally located in the nucleus, where it has been shown to be involved in transcription, RNA splicing and transport [40]. In mutations associated with ALS, there is a shift of TDP43 from the nucleus to the cytoplasm, and the normal nuclear TDP43 staining is lost, together with a propensity to aggregate [41]. Finally, mutations in fused in sarcoma/translated in liposarcoma (FUS/TLS) gene, that encodes for another RNA processing protein, is another cause of familial ALS [42, 43]. Importantly, these two types of mutations led to the hypothesis of RNA processing abnormalities as a cause of ALS [42, 43].

10.3. ANIMAL MODELS OF ALS

Rodent models expressing mutants forms of SOD1 represent the most studied and used models at present. Shortly after the discovery of SOD1 mutations in FALS

[17] a transgenic mouse model ($SOD1^{G93A}$) of SOD1–ALS was developed, expressing approximately 20–24 copies of the human coding sequence with the G93A mutation, under control of the human SOD1 promoter [21]. Since the development of this model, over twenty other SOD1 models have been created and SOD1 transgenic rodents have been used as the primary models for studying ALS. The animals present with an adult-onset progressive paralysis, characterized by loss of motor neurons in the ventral horn, axonal and mitochondrial dysfunction, muscle denervation, and astrocytic and microglial activation [21-26]. Overexpression of mutant SOD1 is not limited to mice, as transgenic rats have also been developed that recapitulate many features of ALS, resembling those described on mice [25, 44, 45].

In order to rule out the possibility that the disease phenotype may be the result of overexpression of SOD1 *per se*, lines of transgenic mice overexpressing the human wild type protein have also been created [21, 46]. Although animals appear to undergo a multisystem dying-back axonopathy, no lines of transgenic wild-type SOD1 mice have succumbed to ALS-like symptoms to date [47, 48].

Following the identification of ALS causative mutations in the gene TARDBP encoding TAR DNA-binding protein 43 (TDP43), knock-out mice and mice overexpressing wild-type or mutant forms of TARDBP were developed to determine whether mutations in TARDBP cause ALS due to a loss-of-function or gain-of-function mechanisms [49-54]. However, even if some of these new animal models of ALS present motor neuron degeneration and several studies are being published about the role of non-neuronal cells in the disease, this chapter will only address the mutant SOD1 rodent models.

10.4. NON-CELL AUTONOMOUS MECHANISMS IN ALS

ALS is a non-cell autonomous disease. This concept emerged from important works that showed that motor neuron degeneration is non-cell autonomous and other non-neuronal cells (*i.e.*: microglial cells, astrocytes) actively participate in the degenerative process [reviewed in 1, 55]. These discoveries were possible through transgenic mice that selectively express mutant SOD1 in one exclusive cell type or at the contrary where mutant SOD1 was ablated from a specific cell

type. For example, when mutant SOD1 was exclusively expressed in mouse motor neurons, this was not sufficient to lead to neurodegeneration [56, 57]. Indeed, only after increasing the expression of mutant SOD1 a late effect of motor neuron degeneration could be observed [58]. Moreover, selective expression of mutant SOD1 in astrocytes and microglia also fails to induce motor neuron degeneration [59, 60], even though a recent publication showed that the transplantation of glial-restricted precursor cells expressing mutant SOD1 that differentiate into astrocytes is able to induce the death of motor neurons in rats [61]. These transgenic animals expressing mutant SOD1 in only one cell type, evidence that the contribution of multiple cell types is needed for motor neuron degeneration. Interestingly, an original approach using chimeric mice expressing both wild-type and mutant SOD1 consolidated the non-cell autonomous disease concept, indeed motor neurons expressing mutant SOD1 lived longer when surrounded by wild-type non neuronal cells, delaying disease onset and extending life-span of chimeric mice [62]. On the contrary, decreasing expression of mutant SOD1 in motor neurons led mainly to a late disease onset without affecting majorly disease progression [63-66].

These approaches also permitted to elucidate whether glial cells as well as other non-neuronal cells participate in the degenerating process. I will describe below what is known about the role of astrocytes and microglia in the neurodegenerative process occurring in ALS.

a) Role of Microglia in ALS

Microglia and astrocytes participate on the neuroinflammatory process. Activation of microglial cells and astrocytes occurs in several neurodegenerative diseases including ALS, and is characterized by a phenotypic change involving proliferation as well as morphological and functional modifications (*e.g.*: induction of pro-inflammatory molecules). There is a marked activation or proliferation of microglia and activation of astrocytes in ALS patients [67] as well as in mouse models of ALS [68, 69].

Microglial cells, are the macrophages of the CNS, and were originally described by del Rio Ortega, a Cajal student, in 1919, and further described by Penfield in 1925 [70]. Microglia cells are the intrinsic immune effector cells of the central

nervous system (CNS) [71]. Under physiological conditions "resting" or as proposed by Hanisch & Kettenmann [72] "surveying" microglial cells are actively screening their microenvironment in order to maintain the normal function of the CNS [72-75]. Importantly this "resting" or "surveying state" is an active state and involves besides the continuous scanning of their environment the constant communication with surrounding cells of the CNS. The chemokine fraktalkine, and its receptor CX3CR1, as well as CD200-CD200R, and SIRPα-CD47 actively maintains microglial cells in a resting state [76-78]. Upon damage or stress to the CNS, microglia become activated, a response that normally participate in repairing the altered tissue. However its chronic persistence can be deleterious and participate in the degenerative process of many neurodegenerative diseases including ALS [79]. Microglia response to an activating signal is heterogeneous and depends on the nature of the stimulus, and generally includes phagocytosis, release of neurotoxic factors such as nitric oxide and superoxide, release of pro-inflammatory or anti-inflammatory cytokines, neurotransmitters as well as neurotrophic factors [72, 80].

Microglial activation is well documented in both ALS affected areas from patients and animal models of ALS [68, 81-83]. Intense microglial activation has been broadly demonstrated already at early stages of the disease and increases with disease progression up to end-stage in mouse and rat models for ALS [68, 83-87]. Turner *et al*. [67] brought *in vivo* evidence of microglia activation. In this paper the authors detected diffuse cerebral microglial activation *in vivo* during the progression of the disease in ALS patients by PET imaging coupled to [11C](R)-PK11195 a ligand for the ''peripheral benzodiazepine binding site'' which is expressed by activated microglia. They showed evidence of diffusely increased microglial activation in both motor and ''extra-motor'' cerebral regions in a small subset of ALS patients compared to control subjects [67].

An important role for microglia in ALS was depicted in studies using a genetic approach in mice bearing the $SOD1^{G37R}$ or $SOD1^{G85R}$ mutation. Decreasing the expression of mutant SOD1 specifically in macrophage/microglia altered the progression of the disease by increasing the late disease phase and overall survival [64, 66]. Similar results were obtained using an alternative technique to replace mutant microglia with wild-type microglia and study their involvement in motor

neuron disease by using PU.1 knock-out mice, which lack macrophages, B and T cells, neutrophils and microglia [60]. They were able to show first, that after bone marrow transplant derived from a $SOD1^{G93A}$ mice to PU.1-/- mouse, mutant microglia were not able to induce motor neuron degeneration. Second, while transplanting WT bone marrow to double mutant $SOD1^{G93A}$/PU.1-/-, mice replenished their spinal cord with wild type microglia, and showed an extended survival which was due to a prolongation of disease duration, as onset was not altered. However it remains to be clarified if other immune cells participate in this increased survival, as T cells were shown to modify ALS pathology.

b) Role of Astrocytes in ALS

Important publications of the last years shed light onto astrocyte's role in motor neuron degeneration [65, 88-91]. Astrocytes were first shown to display a decreased expression of glutamate transporter GLT1 [25, 28, 92] favoring glutamate-induced excitotoxicity in ALS. Further evidence for a role of astrocyte in ALS came for experiences of astrocyte cell cultures carrying $SOD1^{G93A}$ showing toxicity towards isolated motor neurons [88, 89, 93]. *In vivo* evidence for an active role of astrocytes in ALS pathology came from decreasing the expression of $SOD1^{G37R}$ specifically in astrocytes, which delays the progressive phase of the disease and increases survival of mutant SOD1 transgenic mouse [65]. Conversely, increasing astrocytes' antioxidant defenses by overexpression of the transcription factor Nrf2 extended the lifespan of $SOD1^{G93A}$ mice [90]. Moreover, transplantation of lineage-restricted astrocyte precursors into cervical spinal cord delayed progression of mutant SOD1-mediated disease after onset, which highlights the role of astrocytes on disease progression, and support a future for cell therapies [91].

c) Role of Other Non-Neuronal Cells

Whereas motor neuron expression of mutant SOD1 determine the onset of the disease, microglia and astrocyte expression determine the progressive phase of the disease. However, I will briefly mention the role of other cells that have been shown to participate of the degenerative process in ALS.

Schwann cells, the peripheral myelinating cells, have been shown to participate on ALS pathogenesis. Decreasing the expression of the dismutase active $SOD1^{G37R}$

specifically in Schwann cells accelerated the progression of the disease and was associated with diminished levels of insulin-like growth factor 1 [94]. This apparent contradictory result, suggests that mutant dismutase active SOD1 overexpression, due to its antioxidant enzyme activity, is rather protective than toxic in this cell type. On the other hand, the overexpression of the dismutase-active mutant $SOD1^{G93A}$ selectively in Schwann cells driven by the myelin protein zero (P0) promoter did not affect motor performance nor overall survival of mice [95]. These results suggest that mutant active SOD1 accumulation within Schwann cells is not deleterious (and even beneficial) to motor neurons in ALS mice.

Within the muscle, the role of mutant SOD1 expression in disease is still controversial. Indeed a decrease in the expression of mutant SOD1 in muscle had no consequences on ALS disease onset or progression [96, 97]. On the other hand, the expression of mutant SOD1 selectively in skeletal muscle caused damage to muscle, in a similar way of what is observed in ALS SOD1 disease models [98].

The vasculature is altered in SOD1 rodent models of ALS, with modifications of the blood brain barrier permeability early in disease characterized by loss of tight junctions between endothelial cells, which allow the leakage of potentially neurotoxic blood components and microhemorrhages [99-102]. Importantly, decreasing the expression of mutant SOD1 within endothelial cells does not affect ALS disease course [101]. Finally, blood-spinal cord barrier dysfunction was also reported in ALS patient cases with erythrocyte extravasation and pericyte reductions [103, 104].

T-lymphocytes also play a role in ALS disease. Human ALS cases and mice (mSOD1) tissue showed evidence of T-lymphocyte infiltration [81, 84, 105, 106]. In contrast, B-cells could not be identified nor in the spinal cord of ALS mice [107] nor in human ALS spinal cords [105, 108] and mutant SOD1 mice deficient in B lymphocytes develop ALS in a similar way than control mice [109]. In order to shed light onto the role of adaptive immunity in ALS, different studies with mice were used to show the involvement of T cells in ALS. Firstly, $SOD1^{G93A}$ mice that lack functional CD4 T- and B-cells (RAG2-/- mice) showed an accelerated disease (though opposite results were obtained by Tada *et al*. [110])

that was in part reverted by $SOD1^{G93A}$ or ^{WT} bone marrow transplant, showing that lymphocytes had a beneficial effect on the disease [111]. Similar results were obtained when mutant $SOD1^{G93A}$ mice were bred onto a $TCR\beta^{-/-}$ background (mice that are deficient in T cells), accelerating disease progression [107]. This study also suggests that T cells play an endogenous neuroprotective role in ALS by modulating in microglia a beneficial inflammatory response upon neurodegeneration by increasing the expression of protective factors like IGF-1 and decreasing pro-inflammatory cytokines such as IL-6 [107, 112]. Lymphoid deficits including impaired T-cell function and increased death was described in the $SOD1^{G93A}$ mutant mice [113]. In order to correct them, mutant SOD1 mice were reconstituted with donor lymphocytes showing that T regulatory cells (Treg) and T effector cells (Teff) from wild-type donor mice, but not naïve T lymphocytes, increased mice survival. Tregs were predominantly responsible for increasing the time to disease onset, whereas Teff for decreasing the progression of the disease (increasing the time from onset to death) [113]. Finally, the neuroprotective role of Tregs is supported by studies in ALS patients, where the numbers of Tregs and FoxP3, a transcription factor required for Treg function, were inversely correlated with disease progression rates [112, 114].

Oligodendrocytes are the myelinating cells of the CNS, however, they were recently shown to also provide metabolic support to neurons by the lactate transporter monocarboxylic acid transporter 1 (MCT1) [115, 116]. Oligodendrocytes have been implicated in ALS and MCT1 has been shown to be downregulated in the motor cortex of ALS patients and in the spinal cord of $SOD1^{G93A}$ mice [117]. Oligodendrocytes were shown to be dysmorphic and degenerate in ALS [118, 119]. In response to this, new oligodendrocytes are continuously generated through an increased rate of proliferation and differentiation of NG2 glia/oligodendrocyte precursors. However, their differentiation is affected leading to dysfunctional cells with impaired myelinating capacity [118, 119]. Moreover, selective removal of mutant $SOD1^{G37R}$ from oligodendrocyte progenitors prolonged survival in mice mostly due to a delay in disease onset [119]. Oligodendrocyte's lineage cells are thus affected in both

human patients with ALS and mutant SOD1 mice and may participate on motor neuron degeneration [117, 118].

CONCLUDING REMARKS

Overall these studies permitted to conclude that non-neuronal cells play an important role in the disease, however the mechanisms underlying cell neurotoxicity in ALS are currently unknown. Of note these mechanisms are of invaluable importance for understanding the pathogenesis of ALS and for novel therapeutical intervention during the symptomatic phase of the disease.

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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CHAPTER 11

Multiple Sclerosis: An Overview

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Abstract: Multiple sclerosis is a chronic, inflammatory, immune-mediated disease of the central nervous system. Current evidence indicates that a complex genetic trait associated with environmental factors probably triggers MS. The hypothesis is that the inflammatory response starts when CNS protein-specific CD4+ T cells become activated in the periphery, cross the blood/brain barrier, and induce CNS autoimmunity. A disturbed balance between cells that induce or cause demyelination and regulatory T cells capable of suppressing these auto-reactive T cells underlie MS pathogenesis. Inflammation and oxidative stress are major causes of tissue damage in the CNS. Diagnostic criteria include paraclinical laboratory assessments emphasizing the principle of lesions disseminated in time and space. Cerebrospinal fluid analysis remains mandatory in order to support the diagnosis and differentiate MS from other diseases. Disease modifying therapies (DMT) are available for MS patients like recombinant Interferon β (IFN-β) and Glatiramer Acetate (GA) that present similar clinical outcomes showing reduction in patient's annual number of relapses, MRI T2 lesion load reduction and delay of disability. Recently, a monoclonal humanized antibody, Natalizumab, was re-launched showing a larger reduction in annual number of relapses and MRI lesions in the CNS. Besides, Fingolimod (FTY720) was also introduced as the first oral drug with similar effects. Corticosteroids are the first line therapy for acute MS exacerbations. The parenteral use of Cyclophosphamide, Mitoxantrone and Cladribine may benefit some patients with aggressive disease. Oral immunosuppressive drugs (azathioprine, mycophenolatemofetil and methotrexate) have also been reserved for patients whose disease progression cannot be controlled by DMTs.

Keywords: CSF study, genetics of MS, immunology of MS, multiple sclerosis, MS treatment, MS diagnosis, white matter pathology, brain cortical atrophy, CSF oligoclonal bands, CNS demyelinating disease, EAE model, low vitamin D level, MRI brainT2 lesions, reactive oxygen species, Th17 cells, EDSS scale, neurodegeneration, HLA-related susceptibility, relapsing, secondary progressive phase.

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11.1. MULTIPLE SCLEROSIS: THE DOUBLE EDGED SWORD OF IMMUNE SYSTEM

Multiple sclerosis (MS) is a chronic, inflammatory, immune-mediated disease of the central nervous system (CNS), usually occurring in young adults, and it is more common in females. The inflammatory response in the central nervous system (CNS) may cause the demyelination and loss of oligodendrocytes, neurons, and axons [1]. Traditionally it has been thought that MS is an inflammatory disease primarily localized in the white matter of the brain and spinal cord. However, recent studies have identified gray matter inflammatory lesions in MS patients that appear at the earliest stages of the disease [1, 2].

The incidence of the disease varies worldwide, with a prevalence that ranges between 2 and 150 per 100,000 depending on the specific population. There are few reliable epidemiologic studies about MS in Latin America [3, 4]. However, the number of new cases is increasing especially in the southern area of South America. Clinical symptoms of MS depend on the site of neurologic lesions. The first relapse of the disease is designated clinically isolated syndrome (CIS). Usually, CIS is followed by a relapsing and remitting course (RRMS), which is characterized by recurring attacks or exacerbations of existing deficits (relapses) followed by partial or full recovery (remission). After approximately 10 years, half of these patients convert to the secondary progressive (SPMS) phase of the disease, in which there is acceleration of disability, with continuous progression of the neurologic deficits. A fewer percentage (10%) of MS patients will present a progressive course since the beginning, which is called primary progressive MS (PPMS).

According to 2010 revisions to the McDonald Criteria, magnetic resonance imaging (MRI) of the CNS can support, supplement, or even replace some clinical criteria [5]. MRI findings could be integrated to the clinical presentation to demonstrate dissemination of lesions in both space and time. Based on work of the European MAGNIMS research group, dissemination in space (DIS) can be demonstrated with at least one T2 lesion in at least 2 of 4 locations (juxtacortical, periventricular, infratentorial and spinal cord) with lesions within the symptomatic region excluded in patients with brainstem or spinal cord syndromes [5]. Dissemination in time (DIT)

can be demonstrated with the appearance of a new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI compared to a reference or baseline scan performed at initial clinical event irrespective of the timing of its acquisition [6]. Another DIT criterion is that the simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions at any time on the baseline MRI can substitute the following-up scan to confirm it [6].

11.2. PATHOLOGY OF MS

11.2.1. White Matter Pathology

In terms of pathology, patients with MS demonstrate white and gray matter lesions. The white matter plaques are usually located in the subcortical or periventricular white matter, optic nerve, brainstem and spinal cord, characterizing the classical MS pathology [7]. The plaques arise from an intense inflammation, demyelination, gliosis, and axonal injury. The focal inflammatory demyelinating lesions are characterized by perivascular infiltrates of CD8+ T lymphocytes, CD4 T lymphocytes [8, 9] gamma delta (γδ) T lymphocytes [10] monocytes and few B cells and plasma cells. Moreover, in the active lesions, macrophages containing myelin debris, complement's components and immunoglobulins are observed [11]. The inflammatory response in RRMS correlates with gadolinium-enhancing MRI lesions, while the inflammation in SPMS phase of the disease has no MRI lesions [12]. In the SPMS, a diffuse inflammation of normal appearing white matter and extensive axonal injury are observed [13].

11.2.2. Grey Matter Pathology in MS

Abnormalities of the cortical gray matter (GM) can be found since the earliest phase of the disease [14-17] and evolve with its progression to the secondary phase of MS [18]. Numerous studies have demonstrated that the changes in GM are related to both physical disability and cognitive impairment [19-22]. Cortical lesions occur early in CIS and relapsing-remitting MS, as well as in PPMS. Lesions increase in number and sizes through the progression of the disease [23, 24]. According to previous pathological studies, GM lesions comprise 26% of all lesions identified in the central nervous system (CNS) [25], and more frequently, they have been found in the frontal and temporal cortex, affecting the motor (30-40%) and cingulate areas

(10%) [26]. Also the subcortical GM could be affected, more frequently encompassing the thalamus, basal ganglia, hypothalamus, hippocampus, cerebellum and spinal cord [16-21]. Mechanisms such as Wallerian degeneration secondary to demyelination and axonal transection, damage caused by reactive oxygen species and nitric oxide or energy failure from mitochondrial are among the factors currently attributed to cause the neurodegeneration observed in MS [27-29]. Although inflammation is less pronounced in GM than in the white matter, its damaging lesions mediated by the immune response cannot be excluded. A recent study demonstrated the presence of CD8 T lymphocytes in perivascular space in the GM's biopsy of MS patients [2]. Moreover, T-cell mediated autoimmunity directed against contactin-2, which is present specifically within the GM, was also identified as a factor contributing to the GM pathology [30].

11.3. GENETICS AND ENVIRONMENTAL RISK FACTORS RELATED TO MS

The etiology of MS remains unknown. However, it is highly unlikely that the disease results from a single causative event. Current evidence indicates that a complex genetic trait associated with environmental factors probably triggers MS. The risk factors may act many years before the development of the disease [31]. Genetic epidemiological studies indicate that the genetic susceptibility must be an important condition to start the disease. Familial aggregation studies showed that the risk of developing MS is higher for people with family member's cases, especially for first-degree relatives (10-25 times). Moreover, monozygotic twins of MS patients have more than 100 times higher risk of developing the disease when compared to the general population [32].

MS is more common in women than men. However, up to now, no relevant MS-associated gene has been found in the X chromosome [33]. The increased risk in women may be related to the female physiology or a higher susceptibility to environmental factors. The female to male ratio for MS incidence ranges up to 3:1 worldwide. This ratio seems to have increased during the last decades. However, the greater incidence in females does not mean a more aggressive disease course or poorer outcome in this gender. Instead, males exhibit a shorter time and a younger age for conversion to the secondary progressive MS and to a faster GM

atrophy and cognitive dysfunction. Thus, disease progression and neurodegeneration are faster in men [34]. The obvious candidate to explain this heterogeneity would be the hormonal difference between the genders. However, no conclusive results have shown a strong correlation of hormones and the incidence or progression of the disease so far. In addition, the increase in the sex ratio for MS over the past suggests that MS susceptibility could be influenced by sex-specific correlation of genetic-environmental interactions.

The high discordance rate among monozygotic twins, the north to south gradient of MS incidence and, especially, the migratory studies indicate the potential environmental influence to MS development. The first and maybe the strongest evidence for a role of environmental factors influencing MS are the migratory studies pointing to different risks of developing MS regarding the MS prevalence of the site you arrive to live. In general, people who migrate to one area before 15 years of age will have the same risk to develop MS as the population of that area. If people migrate after 15 years of age, they will have the risks of their original site. Although the role of many environmental factors has been studied in the last decades, only infections, latitude/vitamin D and certain social behavior (*e.g.*, smoking habit) were related to increased risks of MS development [35].

Sunlight exposure and serum vitamin D levels are the most likely explanation for the association of MS with world's latitude. The main source of vitamin D is UV radiation exposure (~95%) and only a small part is given by food intake (~5%). There is a strong correlation between sunlight exposure and MS incidence worldwide. Although the minor part of vitamin D is given by food intake, some studies show a decrease of the MS risk in population with a vitamin D rich diet. For example, in Norway, the incidence of MS is higher inland compared to the seaside population. The most suitable explanation is the high consumption of fish oil by the seaside population, which is rich in vitamin D [36]. Furthermore, mothers who have lower sunlight exposure during the first three months of pregnancy give birth children with a higher risk developing MS later in life. Although the mechanisms by which vitamin D can be beneficial to MS incidence and/or progression are not completely elucidated, many studies have shown a preferential immunomodulatory action of vitamin D. Many studies were done in the animal model experimental autoimmune encephalomyelitis (EAE) of MS.

Studies have shown that treatment with vitamin D, or its active form 1,25-dihidroxy vitamin D (1,25-OH Vitamin D), induces tolerogenic dendritic cells (DCs) and regulatory T cells. These regulatory T cells modulate the proinflammatory T-effectors cells (Th1 and Th17) [37, 38].

The natural question is if vitamin D supplementation attenuates MS activity. The current studies have shown a beneficial effect of vitamin D supplementation, but these are small and not controlled. Interestingly, the last International MS Genetics Consortium study has confirmed a significant polymorphism of vitamin D receptor in MS patients. The polymorphic vitamin D receptor may result in poor vitamin D assimilation. Indeed, the best results from clinical trials came from high-dose supplementation studies [38].

Infections are among the most studied and biologically plausible environmental factors related to MS pathogenesis, especially viral infections. The most accepted theory is that some infectious agents may present a molecular mimicry with myelin compounds. Prominent candidates have included measles, rubella, mumps, herpes simplex virus (HSV) 1 and 2, varicella zoster virus and Epstein Barr virus (EBV). Between those viruses, only EBV appears to have a strong relationship to MS. Many studies have shown that people with MS are more likely to be EBV seropositive (99%) than healthy controls (85-95%), suggesting that the disease may be triggered by a prior EBV infection, although this link remains controversial. Interestingly, the timing of infection seems to play a role in MS incidence. Pediatric cases of EBV infection present a weaker correlation with MS. However, infections during adult life, particularly those associated with clinical infectious mononucleosis (IM), shows associations with a four-fold in MS risk, presenting a mean interval to MS onset of 14 years after IM [39].

The increase of MS incidence in the last century has also pointed out the possibility of social behavior being a contributing factor to MS. This includes diet, pollution, smoking habit, trauma, chemical agents, organic solvents and various occupational hazards. Smoking and obesity are the most studied social behavior factors. Although it is highly improbable that smoking alone would account for the worldwide variation of MS prevalence, individuals who carry HLA-B1*1501 and smoke, presented a higher risk to develop MS. This relationship seems also to be dose-dependent to MS risk.

Ethnic origin appears to play a role in the MS incidence. MS is more common in Northern Europe, Canada and the USA, especially in Caucasians. African Americans, Native Americans, South Americans and Asians have lower incidence of MS. A genetic association of certain genes related to the major histocompatibility complex (MHC) with MS has long been known. The first genetic risk factor discovered was HLA region during the 1970's. The strongest association was first found with human leucocyte antigen (HLA) DR2 isotype, which was lately better refined with DNA-based typing methods to the DRB1*1501. Each copy of this allele increases MS risk around three-fold in the European population. Recent studies have pointed out five alleles in three different loci in HLA region: the HLA-DRB1*1501, *0301 and *0801 alleles, the HLA-A*0201 allele and the HLA-DPB1*0301. Theses alleles could represent changes in MS risk between 26 to 200%. On the other hand, protection from disease may be associated to some HLA class II alleles [40-43]. Many years after the discovery of the DR2 allele, other non-HLA genes were also related to MS (*e.g.* interleukin-7 receptor α, interleukin 2 receptor α, C-type lectin-domain family 16 member A, CD58, tumor-necrosis-factor receptor superfamily member 1A, interferon regulatory factor 8 and CD6). In 2011, the largest genome-wide study so far made by the International MS Genetics Consortium evaluated 9772 MS cases and 17376 shared controls. This study ended up with more than 1 million single-nucleotide polymorphism (SNPs), confirmed the association of 23 previously reported loci and identified 29 new candidates [44]. Most of these genes have an immune function or are involved in immunological pathways and many of them were also described in association with other autoimmune diseases. These results reinforce the existence of similar mechanisms between some, if not all, autoimmune disease, as it is not rare to find MS patients with other autoimmune diseases. Although the knowledge about genetic risk factors has increased enormously in the last decade, some gaps still need to be filled, for example, how genetic factors influence the age of onset, the evolution and severity of the disease.

11.4. IMMUNE RESPONSE IN MS

The classical description of MS pathology involving perivascular immune cells infiltrating the CNS has long supported an autoimmune disease etiology for MS. Moreover, the ability of immune directed therapies to favorably impact MS patients emphasizes the pathogenic role of immune responses.

To avoid autoimmune reactions, auto reactive lymphocytes have to be deleted or rendered tolerant [45]. Several mechanisms are involved in the central and peripheral compartments to induce and maintain tolerance. Defects in these mechanisms are associated with the activation of immune responses against auto-antigen. Central tolerance occurs in the thymic medulla through depleting self-reactive T-cell colonies by negative selection (clonal deletion) [46] and producing $CD4^+$ natural regulatory T cells (nTregs) [47-49].

The Aire gene in mTECs regulate, in part, the presentation of peripheral tissue-specific self-antigens, so-called "promiscuous gene expression", contributing for efficiency of negative selection [50]. A previous study has demonstrated that AIRE-deficient mice showed an earlier development of myelin oligonucleotide glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) indicating that abnormalities in the induction of central tolerance may be decisive to the development of autoimmunity in the CNS [51]. To control immune responses to auto-antigen that are not expressed in the thymus or may escape negative selection, different mechanisms of tolerance are involved in the periphery during the entire lifespan. Mechanisms of peripheral tolerance include cell death with consequent clonal deletion, development of a state of T cell unresponsiveness, and active suppression mediated by Tregs. Dendritic cells (myeloid or plasmacytoid) producing immuno-modulatory cytokines IL-10 and TGF-β or the expression of the tolerogenic molecules indoleamine 2,3-dioxygenase (IDO) or ILTs can regulate several of these processes [52-53].

The focal demyelinating plaque in active MS lesions is formed by inflammatory immune cells such as T and B lymphocytes, activated macrophages and microglia [54]. The idea of MS as a CD4+ T lymphocyte disease has been reassessed, and the role of CD8+ T cells, B cells, and innate immunity has been emphasized. The hypothesis that CD4+ T lymphocyte can initiate the inflammatory response in MS is confirmed by observations from the EAE model. EAE can be transferred to naïve mice through implantation of CD4+ T cell from a diseased animal. CD4+ T cells reactive to many CNS proteins, including myelin associated glycoprotein (MAG), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP), can be isolated either from MS patients or healthy individuals [55]. CNS protein-specific CD4+ T cells become activated in

the periphery, cross the blood/brain barrier, and induce CNS autoimmunity [55]. However, recent studies have shown that dendritic cells and macrophages containing residues of myelin were found in the lymph nodes of patients with MS, showing that cells containing myelin also leave the CNS and sensitize T lymphocytes in the periphery [56].

In addition to the cytokines produced by Th1 or Th17 lymphocytes, products of other cells such as osteopontin (OPN) may contribute to inflammatory response in MS. An increasing body of evidence suggests that OPN may play a role in the pathogenesis of MS [57], inducing cells that cause demyelination (auto reactive effector T-cells, mainly Th1 and Th17) and down regulating regulatory CD4+ and CD8+ T cells that are capable of suppressing these auto-reactive T cells [58]. Patients RR-MS have shown a diminished T regulatory function [59].

Besides the effector role of CD4+ T lymphocytes, CD8+ T cells also contribute to pathogenesis in MS, although the precise role of these cells remains to be elucidated. CD8+ T cells have been identified interacting with antigen presenting cells mainly at the margin of chronic and active lesions [60]. MHC class I proteins are expressed within the MS lesion on astrocytes, oligodendrocytes, and neurons, which suggests that CD8+ T cells could directly interact with these cell types within the CNS [61-63]. Activated CD8+ T cells have been observed within the CNS tissue and CSF of MS patients [64], and analysis of some cases of acute MS have shown granzyme B-expressing CD8+ T cells close to oligodendrocytes or demyelinated axons. In addition, a classical study demonstrated the existence of CD8+ T cells specific for many CNS proteins such as MBP, MAG and PLP in MS patients [65]. CD8+ T cells specific to myelin antigen are capable of killing neuronal cells and releasing proinflammatory cytokines such as TNF-α and IFN-γ, suggesting that these cells may participate in tissue damage in the CNS. Recently, the presence of inflammatory response with increasing number of perivascular CD8+ T cells was described in the gray matter of patients MS with clinically isolated syndrome (CIS), suggesting the contribution of these cells to a destructive CNS immune response even in the early phase of the disease [2].

B cells are also proposed to play a dual role in the pathogenesis of MS. They contribute to the induction of the autoimmune response but also mediate the

resolution of the CNS inflammatory infiltrate [66]. Besides producing autoantibodies, B cells can function as professional antigen-presenting cells, able to activate CD4+T-cell-specific responses through MHC class II, which contribute to the inflammatory response in MS. Moreover, B cells also influence the T-cell response through cytokine and chemokine production [67]. Increase of both B-lymphocyte and plasma cell in CSF correlates positively with lesions in MS [68]. Pathologic studies showed the presence of lymphoid neogenesis, ectopic B-cell germinal center structures, in the meningeal space of SPMS patients and this observation indicates a more severe course of the disease. Moreover, a gradient of neuronal loss in the cortical layers were found associated with these B-cell aggregates and subpial cortical lesions, suggesting that release of soluble factors across the inflamed meninges may cause cortical damage [69]. In addition, infections in B cells may contribute to perpetuate the inflammatory response in MS. Some studies demonstrated the presence of latent EBV infection in a high percentage of brain-infiltrating B and plasma cells in MS [70], particularly, in meningeal B-cell follicles where viral reactivation was observed.

While the adaptive immune response initiates autoimmune inflammation, innate immune cells are critical for sustaining the response that leads to pathology or the repair of tissue damage caused by the inflammatory response [71, 72]. Various cell types that compose the innate immune system share antigen recognition ability through their receptors that do not undergo rearrangement and have no immunological memory. Cells in innate immune response such as phagocytic cells (neutrophil, macrophages, glia and dendritic cells), mast cells, γδ T lymphocytes and natural killer cells participate actively in the inflammation of central nervous system.

Dendritic cells (DCs) act at the interface of innate and adaptive immunity, playing an essential role in the initiation of effective T cell-mediated immune responses. Paradoxically, DCs also have the potential to exert powerful negative regulatory effects on the immune system [73]. Among innate immune cells, DCs are uniquely specialized to acquire, process, and present antigens to elicit helper/effector T cell responses *via* MHC-peptide/T-cell receptor (TCR) interactions and concomitant co-stimulatory signals (B7/CD28). DCs are the only professional APC that can prime naïve T cells and cross-present endocyted antigenic peptides on both MHC class I or class II molecules.

Although *in vitro* experiments have shown that DCs in the CNS could arise from resident microglia in the presence of growth factor, several studies have been demonstrated the transmigration of DCs from blood into the CNS. This cell population is usually not present in the healthy brain, however, they have been found to accumulate in the CNS parenchyma during inflammatory response [74] and they are emerging as important players in CNS autoimmunity, specifically in MS. Indeed, microglia and astrocytes were initially regarded as local antigen presenting cells of the CNS [75-76]. However, mature DC markers have been consistently found in the inflamed meninges and perivascular cuffs of most active MS lesions examined. The identification of dendritic cells in the CNS of patients with MS [77] suggests that macrophages and dendritic cells that enter the CNS by crossing the blood-brain barrier are important antigen presenting cells for *in situ* restimulation and full activation of auto reactive T cells [78]. This strongly supports the view that DCs participate in neuroinflammatory autoimmune responses.

11.4.1. Reactive Oxygen and Nitrogen Species - Intracellular Products Causing Tissue Damage in MS

Inflammation and oxidative stress within the central nervous system are important players on thr chronic tissue damage in MS. Invading inflammatory cells, as well as resident central nervous system cells, produce a number of reactive oxygen and nitrogen species, which cause demyelination and axonal loss [79].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as part of normal cellular physiology. However, the overproduction or a failure of antioxidant mechanisms of these species cause damage to lipids, proteins and nucleic acids, leading to cell death (for additional information on this topic please see Chapter 7). Neurons are constantly exposed to low levels of these oxidative/nitrative species which rapidly may induce repair and protection mechanisms. During inflammation, however, these defenses may not be sufficient to neutralize the oxidative/nitrative stress (*e.g.* superoxide ions, hydrogen peroxide, nitric oxide, and peroxynitrite) and damage to the cells may occur, contributing to tissue damage in MS. High levels of NO, peroxynitrite, and superoxide have all been demonstrated in spinal fluid from patients with MS [80]. Human microglia is one of the most potent producers of superoxide [81], and these cells are activated and recruited during inflammatory demyelinating to

lesions within the CNS. There is also evidence for increased ROS production in EAE, especially by macrophages and microglial cells, and higher levels of superoxide affecting the brain areas. It has also been described that peroxynitrite is formed very early in the course of EAE and correlates with disease activity. In addition, ROS also may cause neuronal mitochondrial dysfunction, which may lead to cell death, as shown in some neurodegenerative diseases, including MS. Mitochondria are essential to neuronal viability and function, throughout their roles in ATP production and intracellular calcium regulation among others [82].

Oligodendrocytes are susceptible to ROS-mediated damage at levels which generally do not affect other cells such as astrocytes or macrophages. The iron found in oligodendrocytes in high levels may react with hydrogen peroxide contributing to the formation of the highly toxic peroxynitrite, which may explain this susceptibility of oligodendrocytes. Furthermore, hydrogen peroxide produced in peroxisomes and accumulated in oligodendrocytes contribute to the failure of long-term repair of myelin and to the axonal loss associated with the progressive phase of MS. In addition, pre-oligodendrocytes appear to be significantly more sensitive to oxidative stress compared to mature oligodendrocytes impairing further repair and remyelination [83].

Nitric oxide (NO) is produced in the nervous system in response to inflammation through the induction of nitric oxide synthase (iNOS). It has been demonstrated that there is increased iNOS production in the CNS of animals with EAE [83]. iNOS mRNA has been identified in MS plaques and macrophages, astrocytes, and microglia within active MS lesions expressing high levels of iNOS and endothelial NOS. There is also evidence that increased proinflammatory cytokine production in MS, such as TNF-α and INF-γ, contribute to a NO increase. Moreover, higher levels of NO have been demonstrated within the peripheral monocytes of patients with MS. Other reactive species such as nitrite and nitrate levels are elevated in the CSF of patients with MS and peroxynitrite is found in brain areas of demyelination and inflammation.

11.4.2. Beneficial Effects of Inflammation in CNS

The neurotoxic properties of inflammation are thought to be at least partially responsible for the axonal damage observed either in white matter or grey matter

in MS [2, 7]. However, as mentioned before, a number of recent studies have proposed that autoimmune inflammation can have a neuroprotective role in the CNS [84]. Previous studies demonstrated that myelin-autoreactive T cells show neuroprotective effects *in vivo*, and activated antigen-specific human T cells and other immune cells produce neurotropic factors that are involved in the protection of CNS [85, 86] (For additional information, please see Chapter 17). BDNF supports remyelination after both peripheral and CNS injury, and in addition, can downregulate the expression of MHC molecules in hippocampal slices, acting as an immunomodulator agent [87].

Traditionally, neurons have been considered the major cellular source of BDNF in the CNS [88]. However, recent studies demonstrated the production of substantial amounts of BDNF also in immune cells [89]. Actively demyelinating lesions present a higher percentage of BDNF-immunoreactive cells when compared to inactive lesions, in addition to neurons located in the vicinity of these active lesions, as well as reactive astrocytes [90]. This neurotrophin-mediated neuroimmune signalling network could be a major factor that helps to preserve axons in a microenvironment insulted by inflammatory response. Thus, it should be considered as a beneficial aspect of neuroinflammation that could be preserved therapeutically or even reinforced using immunomodulatory treatment regimen.

11.5. CEREBROSPINAL FLUID IN MS

As we have seen in the beginning of this chapter, MS presents a variable clinical presentation and no diagnostic laboratory test. Diagnostic criteria include paraclinical laboratory assessments emphasizing the principle of lesions disseminated in time and space [5]. Cerebrospinal fluid (CSF) analysis remains mandatory in order to support the diagnosis and to differentiate MS from other diseases that can mimic it. The lack of typical findings on MRI and CSF examination should raise suspicion that MS is not present, since very few patients with MS have a normal MRI of the brain or normal CSF [5].

The heterogeneity observed in MS results in delays to the definite diagnosis. Extensive studies are made in the field of biomarkers to improve the diagnostic discrimination. CSF is very often the most accessible material and changes in its

composition may reflect pathological processes of MS such as inflammation, demyelination, neuro-axonal loss, gliosis and regeneration. Even though there is no standard diagnostic CSF test, multiple parameters can indicate the probability of MS. On the other hand, there is no definite relationship between the abnormalities in the CSF routine examination and the clinical course, duration, or severity of the disease [91].

CSF analysis is based on microscopic examination of white blood cells (WBCs), total protein concentrations, glucose level and specific albumin and immunoglobulin measurements. In this way, it is possible to identify CSF-specific cell types (lymphoid cells, plasma cells, activated B cells and polymorphonuclear cells), which are critical in the diagnosis of many infectious and inflammatory neurological disorders. In MS, two-thirds of patients have a normal CSF cell count and a low level of mononuclear pleocytosis is found in one third of the cases [92]. These cells, reach the CNS migrating from the systemic circulation across an inflammation modified are responsible for the intrathecal IgG synthesis (oligoclonal IgG bands. A permeable BBB in the early pathogenesis of MS and intrathecal production of oligoclonal bands are important processes occurring prior to a clinical manifestation of MS [93].

The evaluation of BBB permeability is more precise by CSF/serum albumin ratio, as albumin can be measured in the CSF and it is not produced in the CNS. An intact blood-CSF barrier is associated with a CSF/serum albumin ratio $< 8x10^{-3}$. The albumin quotient is the basis for quantitation of the intrathecal immunoglobulin response. Almost all inflammatory neurological disorders, accompanied by humoral immune reactions, are characterized by IgG synthesis. Intrathecal IgA and IgM synthesis provide additional diagnostic cues in acute inflammatory diseases of the CNS. In this context, various formulas were devised to calculate the intrathecally produced IgG fraction in the CSF [94, 95], which has similar diagnostic sensitivity in association with MS. After the introduction of the empirical ratio diagram [94], which revealed a non-linear relationship between IgG and albumin ratio, a graphic and quantitative illustration of the intrathecal IgG synthesis became possible.

There are two techniques to the detection of intrathecal IgG synthesis in MS. In quantitative analysis (hyperbolic function), each patient is compared with a large

population, whereas in qualitative analysis (oligoclonal bands), each patient is compared with his or her own parallel serum sample. Qualitative analysis is more sensitive for verification of intrathecal IgG production than quantitative ratio diagrams. The consensus for the diagnosis of MS is that isoelectric focusing (IEF) on agarose gels followed by immunoblotting should be the gold standard for detecting the presence of oligoclonal bands [91] **(**Fig. **1**).

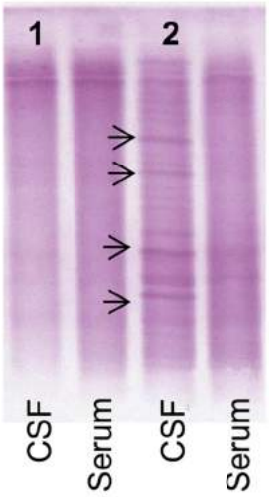

Figure 1: Pairwise cerebrospinal fluid and serum isoelectric focusing on agarose gels followed by immunoblotting of two patients. Number 1shows the absence of oligoclonal bands in CSF and serum. In number 2, we can see oligoclonal bands (arrows**)** restricted to CSF.

The so-called MRZ reaction (measles, rubella, varicella zoster) is even more specific for the diagnosis of MS than formulas that calculate intrathecal IgG production or oligoclonal bands. This reaction reveals the most commonly intrathecally produced virus-specific antibodies and accounts for the presence of a chronic inflammatory disease of the CNS [96].

The following parameters are typically found in MS:

- Mild pleocytosis (WBC 0-50/ mm^3).Very high WBC (>50/ mm^3) is unusual in MS.

- Normal to slightly elevated total protein (up to 90 mg/dl). Low CSF glucose levels (CSF/ serum ratio < 0.4) and very high total protein (>

100mg/ dl) are more consistent with an infectious or neoplastic process.

- Normal to slightly elevated albumin ratio (up to $10x10^{-3}$). The CSF/serum albumin quotient is preferred to detect a blood-CSF barrier (BCB) dysfunction.

- Intrathecal IgG synthesis (qualitative detection). Oligoclonal IgG bands are detectable in 95% of MS cases.

- Intrathecal synthesis of antibodies against measles, rubella and the varicella-zoster virus (MRZ reaction) in 94% of MS patients.

The routine CSF analysis helps to distinguish between other causes of inflammation that mimic MS. Positive CSF findings can be important to support the inflammatory demyelinating nature of the underlying condition. However, it is not specific for MS. Clinical findings and imaging results with the help of positive CSF findings (MS-specific CSF profile) may provide typical parameters to confirm the diagnosis of MS and for a decision in favor of immunosuppressive therapy at an earlier stage of the disease.

11.5.1. Surrogate Markers

Since MS lesions are rarely biopsied, the detection of unusual substances or identification of further parameters in the CSF may serve as surrogate markers. These CSF markers reflect a systemic T-cell proliferation, production of proinflammatory cells in the CNS, activation of inflammatory cells of the CNS, edema, demyelination, remyelination, axonal damage or neuronal atrophy.

The search for biomarkers including those possibly present in the CSF which could predict and assess the course as well as response to treatment in a particular MS patient has not yet been successful. Beyond the oligoclonal bands, no CSF marker has been routinely implemented in the CSF analysis [97, 98]. Promising markers in the CSF by new technologies (proteomic pattern analysis) have so far been investigated but the benefit of these markers for the individual patient still has to be demonstrated [98]. Some findings suggest that CSF proteomic pattern

analysis can increase the accuracy of disease diagnosis of MS-related disorders and will aid physicians in appropriate therapeutic decision-making [99].

11.5.2. Markers for Inflammation and Immune Dysfunction

CSF levels of proinflammatory cytokines (IFN-γ, TNFα, lymphotoxin, IL-4, IL-5) are usually elevated in MS patients. Increased tumor necrosis factor alpha (TNFα) levels in the CSF correlated with the clinical disease activity and gadolinium-enhacing lesions in MRI. Anti-inflammatory cytokines (IL-10, transforming growth factor beta - TGF-β) are up-regulated during periods of remission [100].

11.5.3. Markers for the Alteration of the Blood-Brain-Barrier

Altered concentrations of circulating adhesion molecules and matrix metalloproteinases (MMPs) are indicators of an alteration of the blood-brain barrier [101].

11.5.4. Markers for Demyelination

Elevated CSF levels of MBP and peptides similar to MBP correlated with acute myelin damage in CNS during acute relapses. Autoantibodies against myelin (anti-MOG, anti-MBP, anti-PLP) also belong to this category of markers [102].

11.5.5. Markers for Remyelination

These markers are increased following an exacerbation and the most studied are neuronal cell adhesion molecule (N-CAM), growth factor ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin 3 (NT-3) [103].

11.5.6. Markers for Activation and Damage of Glial Cells

Glial proteins can be detected in CSF when cells are damaged and/or activated. S100b and glial fibrillary acidic protein (GFAP) concentrations are increased in patients with major disability [104].

11.5.7. Markers for Neurodegeneration

Neuro-specific enolase (NSE) levels in the CSF are usually normal in MS patients. However, increased levels of neurofilaments and tau protein

concentrations in the CSF were found in MS patients, revealing axonal damage in the early phase of MS [105].

11.6. MS TREATMENT

Disease modifying therapies (DMT) are available for MS patients. They have in general an anti-inflammatory effect, but only partially control the disease [106]. Recombinant Interferon β (IFN-β) and Glatiramer Acetate (GA) were the first Food and Drug Administration (FDA) approved DMTs and represent the most frequent therapeutic intervention prescribed for patients with relapsing-remitting MS (RRMS). Interferon beta-1b was approved in 1993, GA in 1996, intramuscular interferon beta-1a in 1997, and subcutaneous interferon beta-1a in 2002 [107]. Treatment with GA seems to restore the impaired maturation and altered regulatory function of pDCs and promote a shift between the pro-inflammatory Th1 to the anti-inflammatory Th2 effector cells in MS [108]. IFN-β treatment decreased activated pDCs to produce proinflammatory cytokines (*e.g.* IFN-α, IL-6, TNF-α), and chemokines (*e.g.* CCL3, CCL4, and CCL5) [109]. Also, IFN-β induces restoration of regulatory T-cell function by an increase in newly generated naïve regulatory T cells. This effect may potentially lead to impaired trafficking of activated pDCs to the CNS and, afterwards, attraction of Th1 and Th17 lymphocytes, which would diminish formation of new demyelinating lesions [109].

DMTs present similar clinical outcomes showing approximately 30% reduction in patient's annual number of relapses, MRI T2 lesion load reduction and delay of disability [110-112]. There are subsets of patients that respond different to therapy, which could be related to genetic background that determines differences in cell migration, proliferation, differentiation, antigen presentation and cytokine regulation, and also to appearance of neutralizing antibodies to INF-β [113]. Neutralizing antibodies have the potential to partially or completely reduce drug efficacy, rendering interferon biologically inactive [114].

DMTs are usually safe and well tolerated. The side effects related to INF-β therapy most commonly encountered in clinical practice include redness and burning at the injection site, flu-like symptoms and hematologic abnormalities,

including leukopenia, thrombocytopenia and liver enzyme elevation. Thus, the patients have to perform routine blood studies, including complete blood cell count and liver function tests every 3 to 6 months [115]. Also, the physician should be aware of the possible emergence or worsening of clinical depression with the initiation of IFN therapy. Regarding side effects related to GA therapy, it includes injection site reaction, induration, itching and lipoatrophy. Also, approximately 10% of patients may develop post injection reactions including infrequent episodes of self-limited idiosyncratic chest tightness, palpitations, anxiety, dyspnea and flushing, which usually appears after several months of treatment [115]. Routine blood studies are not necessary during GA therapy.

In 2006, a monoclonal humanized antibody, Natalizumab was re-launched on the market after approval by FDA to the treatment of RRMS, showing approximately 70% reduction in annual number of relapses and 80-90% reduction in MRI lesions in the brain and spinal cord [116]. However, the performances of the different drugs are not directly comparable because of the methodological differences existing in trials and absence of head-to-head comparative data [116].

Natalizumab binds to α4 subunit from α4β1 and α4β7 integrin expressed on activated T cells surface. This blockage avoids the binding of T cells to endothelial receptors VCAM-1 and CAM-1, consequently reducing migration of T cells across the blood brain barrier and their activation by osteopontin and fibronectin, reducing inflammatory response [117]. Neutralizing antibodies against natalizumab could also appear, reducing the drug efficacy and raising the risks of anaphylactic/anaphylactoid reaction during its infusion [117]. In one pivotal trial, two patients developed progressive multifocal leukoencephalopathy (PML), disease caused by JC virus infection [114]. FDA then recommended that Natalizumab should be restricted to selected patients with RRMS, such as those who failed to respond to or do not tolerate other disease modifying therapies, or those who present with a particularly aggressive initial disease course [118]. In one study, the estimated risk of PML was 1 per 1,000 patients treated for an average of 17.9 months (95% CI: 0.2 to 2.8 per 1,000), and this risk may increase with increased exposure time to therapy [119].

Fingolimod (FTY720) was approved by the FDA in 2010 as the first orally administered drug to treat RRMS. Fingolimod is a sphingosine-1-phophate

receptor modulator that inhibits naïve T and central memory T lymphocytes, but not effector memory T cells, to exit lymphoid tissue, preventing auto reactive T lymphocytes from entering the CNS. After phosphorylation, the drug binds four of five subtypes of sphingosine 1 phosphate (S1P) receptors and causes down regulation of the binding of S1P to its receptor. Without this receptor, lymphocytes are retained within the lymph nodes. Fingolimod shows 54% reduction in the annualized relapse rate, 30% reduction in the risk of disability progression and 82% reduction in MRI inflammatory activity [120]. Adverse events include bradycardia following treatment initiation, infrequent and transient first- and second-degree atrio-ventricular conduction block, small increases in blood pressure, hypertension, macular edema and elevation in liver transaminases. There were two fatal infections (disseminated primary varicella zoster and herpes simplex encephalitis) raising questions about susceptibility to herpes virus infection [121] upon treatment. As with all new drugs, post marketing pharmacovigilance will be essential to ensure long-term safety and efficacy [122].

11.6.1 Treatment of a Relapse

Corticosteroids are the first-line therapy for acute MS exacerbations. The largest controlled trial indicating that corticosteroids were effective in MS relapse was the Optic Neuritis Treatment Trial (ONTT). This study showed faster clinical recovery and better visual field results, contrast vision and color vision at 6 months in patients treated with IV methylprednisolone (1g daily for 3 days) followed by an 11-day oral steroid course, than patients treated with oral prednisone alone (1mg/kg day for 14 days), or oral placebo [123]. This trial provided evidence that acute attacks of demyelination should be treated with high-dose corticosteroid rather than low-dose regimens and, therefore, most RRMS trials usually use 1g of IV methylprednisolone daily for 3 to 5 days [107].

Corticosteroids are potent anti-inflammatory drugs reducing edema and aiding in the stabilization of the blood brain barrier. Side effects include elevations in blood glucose, CSF glucose and blood pressure. Patients could present dyspepsia, changes in mood, insomnia, and psychosis. Rarely, corticosteroids can also cause avascular necrosis of femur and susceptibility to infections [107].

11.6.2 Immunosuppressive Drugs

The parenteral use of Cyclophosphamide, Mitoxantrone and Cladribine may benefit some patients with aggressive disease [107]. Mitoxantrone was approved by FDA in cases of rapidly worsening RRMS, secondary progressive MS and progressive relapsing MS, but its use has declined over time because of increased risks to develop therapy-related acute leukemia and impaired left ventricular ejection fraction, leading to congestive heart failure [124].

Oral immunosuppressive drugs have also been reserved for patients, in whom disease progression cannot be controlled by established DMTs or because of serious side effects or specific contraindications to initiate them. The most used drugs are azathioprine, mycophenolatemofetil and methotrexate, with some studies indicating a reduction in the number of relapses from years 1 to 3, and reducing the rate of disability. However, the lack of large clinical trials and head-to-head studies comparing existing DMTs do not support the routine use of these agents [107].

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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CHAPTER 12

Lewy Body Dementia and Frontotemporal Dementia

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Abstract: Dementia with Lewy Bodies (DLB) and Frontotemporal Dementia (FTD) are clinically characterized mainly by gradual progressive impairment of behavior and cognitive functions. The accurate diagnosis of both disorders are very difficult due to significant overlap with other neurodegenerative symptoms. Here, in the chapter, we discuss the last.

Keywords: Dementia with Lewy Bodies, α-synuclein, treatment, biomarkers, genetic association, functional imaging, frontotemporal dementia, FTLD-tau, tau protein, FTLD-TDP, TAR DNA-binding protein 43, FTLD-FUS, fused in sarcoma.

12.1. DEMENTIA WITH LEWY BODIES

Dementia with Lewy bodies (DLB) is a progressive and disabling neurodegenerative disorder affecting the patient's movement, cognition, mood and autonomic function [1]. Although estimates of its prevalence vary from 0-5% of the general population, DLB is thought to be the second most common type of dementia, accounting for 0-30.5% of all dementia patients [2]. DLB was named after α-synuclein protein aggregates, observed in the brain of affected individuals, known as Lewy bodies. Unlike other neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD), DLB was only described in the mid 1990's (although Lewy bodies had been described as early as 1912). In 1996, a consortium first presented diagnostic criteria for DLB [3]. According to a later

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report published by this consortium, DLB should be diagnosed when subjects present dementia plus two or more of the following symptoms: fluctuating cognition with pronounced variations in attention and alertness, recurring visual hallucinations that are typically well-formed and detailed, and spontaneous features of Parkinsonism [4]. Suggestive features may include rapid-eye movement sleep behaviour disorder, severe neuroleptic sensitivity and low dopamine uptake in the basal ganglia, as demonstrated by neuroimaging.

Performing a differential diagnosis of DLB is difficult due to significant overlap with AD and PD symptoms, most notably a drop in cholinergic neurons (observed in AD) and dopaminergic neurons (observed in PD) [5,6]. However, performing an accurate diagnosis is crucial to establishing a correct prognosis and likely clinical course for the patient – especially given that subjects with DLB, as opposed to AD or PD subjects, tend to present hypersensitivity to antipsychotic drugs affecting the dopaminergic and cholinergic systems, rendering the treatment of psychotic symptoms in DLB extremely difficult [7]. Finally, accurate diagnosis is also important for patient/family counselling as well as recruitment for clinical trials of new therapies.

Careful cognitive assessment may lead to differential diagnosis between DLB and AD. As opposed to subjects with AD, DLB patients present consistent impairment of attention/executive functioning and/or visuo-spatial functioning, yet language and memory are often mostly preserved [8]. There may also be differences regarding memory impairment, which, while common in AD, do not seem as prominent in early-stage DLB [9]. Results suggest that the best model for differentiating DLB from AD in early-stage dementia includes visual hallucinations and visuo-spatial/constructional dysfunction, but not extrapyramidal signs [10].

Clinical management of DLB patients is further complicated by their neuropsychiatric profile and extrapyramidal signs. Management should focus on establishing an accurate diagnosis and identifying the target symptoms that concern patients and caregivers. A four-stage, problem-oriented approach to DLB management has been described previously by Barber [11]. Although there are no specific pharmacological treatments, symptoms may be managed using

medications commonly used for AD and PD patients, such as lowest effective dose of levodopa for Parkisonism and cholinesterase inhibitors for neuropsychiatric and cognitive symptoms [12]. Non-pharmacological strategies for cognitive symptoms include explanation, education, reassurance, orientation and memory prompts, attention cues and targeted behavioural interventions [13]. Other therapeutic strategies – such as electroconvulsive therapy [14], repetitive transcranial magnetic stimulation and transcranial direct current stimulation [15], and deep-brain stimulation, which has been shown to be effective in PD [16] – remain to be tested in patients with DLB.

Considerable uncertainty exists regarding the progression and survival of DLB patients. Some results indicate that the rate of decline and mortality in DLB is similar to that of Alzheimer's disease, while others indicate shorter survival periods for patients with DLB. It is important to note that, as yet, no predictive factors of a more severe clinical course or decreased survival have been identified [1,4].

At the cellular level, DLB is defined by the presence of Lewy bodies and Lewy neurites, which consist of α-synuclein protein aggregates. In DLB, accumulation of α-synuclein is observed throughout the brain, including the cortex. α-Synuclein is an abundant 140-residue neuronal protein, that, under physiological conditions, is found mainly in neuronal presynaptic terminals, close to synaptic vesicles. Levels of α-synuclein are regulated by a balance of synthesis, degradation, and secretion, and a major factor possibly driving the aggregation and neurotoxic effects of α-synuclein is the total protein concentration [17]. Protein accumulation leads to neurotoxic effects such as loss of cholinergic and dopaminergic neurons throughout brain areas. Accumulation of α-synuclein is associated with complex machinery that involves lysosomal function and impairment of autophagic pathways, although it remains unclear whether impairment is a cause or a consequence of this aggregation. Other possibilities are currently under consideration, including mitochondrial damage, caspase activation, lysosomal leakage, fragmentation of the Golgi apparatus, interference with synaptic vesicle transport and function, and interference with gene transcription and signaling. Changes in the cholinergic system have also been observed, such as reduction of post-mortem choline acetyltransferase, a presynaptic enzyme responsible for

acetylcholine synthesis in the brain [18]. Amyloid plaques seem to play an important role in neuronal loss given that amyloid-positive DLB and AD patients present very similar patterns of cortical atrophy in the parahippocampal area and lateral temporal and parietal cortices, whereas amyloid-negative DLB/PDD patients have no significant cortical atrophy [19]. For a more detailed description of amyloid plaques, please see Chapter 8.

An explanation for the cellular aberrations observed in DLB would be mutated or polymorphic genes throughout the human genome leading to dysfunctional proteins. Recent studies have shown familial aggregation [20] as well as a familial form of DLB linked to the segregation of genes on the long arm of chromosome 2 (position 2q35-q36) [21]. As expected, most genetic association studies have focused on proteins associated with synucleins (*GBA*, *SNCA*, *SNCB* and *SNCG*), amyloid plaques (*APP*, *PSEN1* and *PSEN2*) and other genes previously associated with increased risk for AD (*APOE*), However, mutations in these genes explain only a small number of clinically diagnosed DLB patients. Moreover, familial co-segregation with disease was often not obtained for these mutations, resulting in incomplete genetic evidence for pathogenicity [22,23]. Therefore, it is generally accepted that the genetic etiology of DLB is complex, and most probably results from the interplay of genetic and environmental risk factors.

In light of the limited sensitivity of current methods of clinical diagnosis, it is important to establish additional markers that, when combined with clinical assessment, can improve diagnostic accuracy [24]. Although genetic studies have not yet identified any reliable markers, other molecules have been investigated as potential biomarkers of DLB disease state or prognosis. Literature from the past few decades has suggested that routine cerebral spinal fluid (CSF) biomarkers may be effectively used for diagnosing not only AD but also DLB cases. CSF beta amyloid Aβ42, suggested to be inversely related to amyloid plaque deposition density, is significantly lower in DLB patients when compared to controls, but is no different than that of AD patients. It is important to note that, at this time, neither observation has reached the necessary levels of sensibility or specificity that would allow for their translation into clinical practice. Other CSF markers still require further study regarding levels of amyloid Aβ38, α-synuclein, magnesium, calcium, cocaine- and amphetamine-regulated transcript (CART),

DK-1 (coded by PARK7 gene), dihydroxyphenylacetic acid/dopamine and homovanillic acid/dopamine ratios that are markers of dopamine metabolism. Preliminary results with these markers have shown considerable heterogeneity that may be associated with heterogeneous inclusion criteria of subjects (*e.g.* diagnostic criteria, time of illness, age at onset), methodological differences in biomarker quantification, and lack of pathological confirmation and/or underlying genetic differences [22]. Furthermore, studies using non-CSF biomarkers, for instance proteins expressed in blood cells, serum levels and large-scale genomic, proteomic and lipidomic assays, are still incipient while common for other diseases like AD and PD.

Functional imaging studies using single-photon emission computed tomography (SPECT) and positron emission tomography (PET) are important tools to better understand the pathophysiological mechanisms at the root of DLB and its differences from AD and PD. Many neuroimaging studies have revealed severe degeneration of the nigrostriatal dopaminergic circuit, also observed in PD, but not in AD [6]. In fact, dopaminergic alteration is so frequently observed in these cases that lower dopaminergic uptake in the basal ganglia has been included as a suggestive feature for diagnostic purposes, although false positives may occur in patients with forms of Parkinsonism with associated dementia. Another experimental imaging technique uses N-(3-fluoropropyl)-2β-carbomethoxy-3β-(4-iodophenyl)nortropane (FP-CIT), a probe with a known affinity for dopamine transporters. This technique has been reported as greatly improving DLB detection sensitivity [25-27]. Other potential biomarkers include 18F-fluoro-L-dihydroxyphenylalanine ([18F] fluorodopa) PET, which assesses the integrity of the nigrostriatal pathway; 18F-fluorodeoxyglucose [18F]FDG PET, which assesses metabolic deficits; and abnormal MIBG (meta-iodobenzylguanidine) imaging, which assesses sympathetic cardiac denervation [24].

Several other neuroimaging studies have been conducted in view of evaluating functional and structural alterations in DLB patients, although results still require further replication. It has been suggested that, contrary to patients with AD, patients with DLB do not present hippocampal atrophy in magnetic resonance imaging (MRI) or cortical uptake in amyloid PET [8]. DLB occipital lobes seem to present a smaller increase of blood perfusion (hyperperfusion) than AD lobes,

and occipital metabolism in DLB is lower than that of AD and control subjects, although the overall brain metabolism profiles of DLB subjects appear to be comparable to those of their AD counterparts [24]. DLB subjects also present more prominent occipital lobe hypometabolism as compared to FTD patients, who present hypometabolism of the frontal and temporal cortices. Furthermore, compared to Parkinson's disease dementia, DLB patients present reduced anterior metabolism [28]. Studies using structural MRI and functional MRI have indicated that DLB patients present global grey matter atrophy in the temporal, parietal, middle and inferior gyri and occipital lobe, while occipital lobe structure remains unaltered [29].

All things considered, studies on the diagnosis, epidemiology and pathophysiology of DLB are much scarcer than those on more prominent neurodegenerative disorders such as AD and PD. Future directions in research are focused on the genetic background and epidemiology of DLB in various populations, as well as in the improved understanding of biomarkers, pathophysiology and use of neuroimaging. A growing body of clinical and pathological evidence supports the notion that AD, DLB and PD with dementia are different members of the same disease continuum [23]. Thus, a deeper understanding of any one of these diseases would further elucidate mechanisms underlying DLB, with consequent improvement of DLB clinical management.

12.2. FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia (FTD) is clinically characterized mainly by gradual progressive impairment of behavior, personality and/or language. In general, FTD patients display severe deficits in their judgement and insight, decreased interest in their surroundings, verbal and physical aggressiveness, negligence of personal hygiene, hyperorality and stereotypical behavior [30]. These features are grouped into syndromes of symptoms which may overlap as the disease progresses. The two most common syndromes are behavioral variant (bvFTD) and primary progressive aphasia (PPA). PPA can be divided into two language variants: progressive nonfluent aphasia (PNFA) and semantic dementia (SD) [31]. Additionally, there is a significant subset of FTD patients who present Parkinsonism, suggesting the coexistence of FTD and motor neuron disease

(FTD-MND) [32]. FTD patients share many common features with patients of Alzheimer's disease (AD). However, compared to patients diagnosed with AD, FTD patients have relative preservation of memory [30]. Although FTD is less common than AD, it accounts for almost 50% of all cases of dementia diagnosed before age 60. Furthermore, at present, neither disease is curable and the drugs that are currently used for AD treatment lead to severe side-effects when used on patients with FTD [33].

Macroscopically, the neuropathological features of FTD are heterogeneous. In contrast with controls and AD patients, the FTD patients commonly present selective asymmetrical degeneration of the frontal and temporal lobes [34]. This specific pathological characteristic presented by FTD patients is often called 'frontotemporal lobar degeneration' (FTLD) [35].

The genetic and pathological features of FTD are also heterogeneous, however, knowledge of the molecular and neuropathological features of FTD has increased considerably over the past five years. Many studies using animal models have proven very useful in understanding the mechanisms of FTD. Furthermore, thanks to genetic associative studies and especially to pathological studies, we are now able to divide FTLD into three main subgroups, based on the abnormal intracellular accumulation of a disease-specific protein:

A. FTLD-tau, which presents aggregation of hyperphosphorylated tau protein;

B. FTLD-TDP, which presents ubiquitination of TAR DNA-binding protein 43 (TDP);

C. FTLD-FUS, which is associated with mutations of the fused in sarcoma (FUS) [35].

Nevertheless, better molecular and biochemical characterization of FTD will help in the development of tools for better diagnosis and pharmacological treatment. In this chapter we will review the clinical, genetic and neuropathological features of FTD and its subgroups.

12.2.1. FTLD-tau

Several studies have demonstrated a strong genetic component to FTD. Family history of FTD is present in 25-50% of cases, which suggests a strong hereditary factor for the disease. At the moment, certain mutations in different genes, such as two genes in chromosome 17 – namely, progranulin (*PGRN*) and microtubule-associated tau (*MAPT*) genes – are well investigated [36]. The *MAPT* gene is located on chromosome 17q21.1 and many studies have shown that *MAPT* mutations are linked to 5-20% of cases of familial FTD. Imaging studies have demonstrated an association of *MAPT* mutations and morphological alterations in FTD patients. For example, patients with *MAPT* mutations present focal temporal lobe atrophy. However, there are variations between different *MAPT* mutations and the morphological alterations in the brain [37]. Interestingly, genetic associative studies also demonstrate associations between mutations in the *MAPT* gene and clinical features of FTD. For example, *MAPT* mutations were shown to be associated with bvFTD [31]. Furthermore, as mentioned previously, some FTD patients display symptoms of Parkinsonism, and genetic associative studies have demonstrated correlations of *MAPT* mutations with FTD comorbidities. For example, both intronic and exonic mutations were identified in *MAPT* in FTD patients with Parkinsonism.

At first, due to different studies having shown an association of mutations in chromosome 17 with FTD with Parkinsonism, this subgroup was known as FTDP-17. However, after a better characterization of the subgroup, the gene *MAPT* was implicated in the disease. This gene encodes the tau protein and, consequently, this subgroup was renamed and is now known as FTLD-tau. It is the best understood of the subgroups.

Tau is a protein with multiple serine and threonine residues which can be phosphorylated by many kinases. Physiologically, tau is localized mainly in the axons and its function is to stabilize the microtubules through its C-terminus binding domain. Additionally, through the N-terminus projection domain, it regulates the microtubules' interaction with other proteins of the cytoskeleton as well as with proteins in the membrane [38, 39]. The brains of FTD patients present neurofibrillary lesions and neuronal and synaptic loss at specific sites. The

neurofibrillary lesions are found in both cell bodies and apical dendrites, and contain neurofibrillary tangles (NFTS). These NFTS are aggregates of the hyperphosphorylated protein tau in neurons and glia [40]. When it is hyperphosphorylated, tau dissociates from cellular microtubules. The dissociated hyperphosphorylated tau forms aggregates that are deposited inside of neurons as NFTs. Depolymerisation of the microtubules is also present [41].

Today, many of the mutations found in FTLD-tau patients have been replicated in biological models for scientific studies, such as transgenic mice, flies and *Caenorhabditis elegans*, helping in the understanding of the biological mechanisms involved in this pathology [42] (for additional information see Chapter 3). For example, transgenic mice expressing the human FTD mutant P301L tau, which is the longest brain tau isoform containing exons 2 and 3 as well as four microtubule-binding repeats, have been shown to display hyperphosohorylation of tau, aggregates and NFT-formation [43]. Other studies have demonstrated that mice expressing the human P301L tau only in oligodendrocytes and astrocytes present neuronal impairments and axonal degeneration [44]. Furthermore, Drosophila melanogaster and *C. elegans* expressing mutant tau also present behavioural alterations, synaptic abnormalities and neuronal loss [45,46]. Taken together, these studies demonstrate that the mutations in chromosome 17 associated with FTD cause neurodegeneration.

12.2.2. FTLD-TDP

FTLD-tau is the best understood of all the FTLD subgroups. However, most cases of FTD are characterized by the pathological ubiquitination of proteins. Ubiquitination is a post-translational modification that involves a covalent bond between a target protein and an ubiquitin residue. When ubiquitin is added to a protein, it activates cascades that can recycle, destroy or transport the proteins to specific intracellular locations. Because of the ubiquitination of proteins and absence of tau protein aggregation, this subgroup was previously called FTLD-U. This subgroup is sometimes also known as FTDU-17 because of associations of these pathological features with mutations in chromossome 17. However, recent post-mortem studies of FTLD patients have shown inclusions in the hippocampus, substantia nigra, basal ganglia, extramotor cerebral cortex, lower motor neurons

of the brainstem and spinal cord formed by an ubiquitinated form of the TAR DNA-binding protein 43 (TDP-43) [35]. For this reason, after the identification of the TDP-43 as the ubiquitinated pathological protein, the subgroup has been renamed FTLD-TDP. Today we know that, similarly to tau, TDP-43 inclusions are formed by ubiquitinated, hyperphosphorylated and carboxyl-terminally truncated TDP-43 [47].

TDP-43 is a highly conserved protein located mainly in the nucleus. It regulates RNA functions through mRNA stabilization and transportation as well as through splicing control [48]. When TDP-43 is hyperphosphorylated, ubiquitinated or has the N-terminal truncated, it becomes pathological, aggregating and forming inclusions in the cell cytoplasm. However, the effects of these structural alterations in TDP-43 function are still unknown, though studies of patients with FTLD-TDP and animal models suggest that both loss of function and gain of function mechanisms can cause inclusions and cell death. It is also interesting to note that many studies show that the brains of amyotrophic lateral sclerosis (ALS) patients present the same phenotype, also with TDP-43 ubiquitination [49], which would seem to suggest that ALS and FTLD are closely related.

Many of the neuropathological phenotypes mentioned above, such as the TDP-43 positive inclusions, are associated with mutations in the *PGRN* and *C9orf72* genes [35]. The *PGRN* gene is in chromosome 17q21.32 and, so far, 69 *PGRN* mutations were found to be associated with familial FTD [50]. Furthermore, the clinical features bvFTD, corticobasal syndrome (CBS) and progressive supranuclear palsy syndrome (PSPS) are also associated with *PGRN* mutations [31]. Imaging studies have also shown *PGRN* mutations to be associated with asymmetrical fronto-temporo-parietal atrophy.

The *PGRN* gene codes for the PGRN protein, which is the precursor of granulin (GRN). The PGRN protein suffers a posttranslational process and forms the peptide GRN, which has a neuroprotective function [50]. GRN is a growth factor that is expressed in many cell types, including neurons. It activates intracellular pathways involved in cell differentiation and neurite outgrowth, such as GSK3β and Wnt [51]. GRN also binds to sortilin (SORT1), a receptor for neurotrophic factors in the brain [52]. This interaction, which appears to take place through

endocytosis, modulates PGRN levels in the brain and in plasma. Recent studies have also demonstrated that GRN binds to tumor necrosis factor α receptors acting as antagonist, modulating inflammation [53]. *In vitro* studies suggest that PRGN deficiency might mediate the caspase 3 cleavage of TDP-43, suggesting a link between PGRN and TDP-43 [36].

Currently, we know that pathogenic mutations in the *PGRN* gene cause haploinsufficiency, leading to a decrease of PGRN levels in serum, plasma and cerebrospinal fluid. Many factors that regulate the function of PGRN are also associated with FTD. For example, the microRNAs miR.29b and miR.107 and the uncharacterized transmembrane protein 106B (TMEM106B) regulate the expression of PGRN [35].

Another mutation with a strong association to FTLD-TDP and ALS occurs in the gene *C9orf72*. This gene is located in chromosome 9p and the mutation is a non-Mendelian inheritance of a GGGGCC hexanucleotide repeat located in a noncoding region of *C9orf72* [54]. These expanded 700-1,600 GC-rich repeat units interfere in the protein expression of C9orf72, but the exact minimal repeat size required for the disease manifestation is still unknown. C9orf72 is present in the cytoplasm of neurons and in the nucleus of fibroblasts, but this protein is still uncharacterized and its function remains unknown. There are two different isoforms of the predicted protein – however, the relative expression of each, in different regions of the brain, has not yet been studied. Although these findings support the loss-of-function theory, studies have shown an accumulation of GC-rich transcripts in the frontal cortex of C9orf72 patients. This suggests a possible toxic RNA gain-of-function [54].

The genetic associative studies for the *C9orf72* gene also support the hypothesis that FTD and ALS are closely related. For example, the average *C9orf72* mutation frequencies reported in European and North American populations for familial FTD is 21%, and 37% for familial ALS [35]. However, the clinical presentation of patients with this mutation is heterogeneous and highly variable between and within families. Clinically, FTD patients with this mutation present memory disorder, psychosis, extrapyramidal movement disorder and cerebellar signs. The

symptoms are cumulative with disease progression. Macroscopically, the patients present symmetrical bilateral atrophy, mainly in the frontotemporal regions, but also involving other regions of the brain. However, the unique characteristic feature of patients with this mutation is the presence of neuronal inclusions in the cerebellar granule cell layer, hippocampal pyramidal neurons and other neuroanatomical sites [35] – although several studies failed to observe any atypical distribution or accumulation of C9orf72 in the brains of FTD patients. Furthermore, a recent study showed that this GGGGCC hexanucleotide repeat upstream expansion generates inclusions containing poly-(Gly-Ala), poly-(Gly-Pro) and poly-(Gly-Arg) [55].

12.2.3. FTLD-FUS

The last main subgroup of FTD is FTLD-FUS. FUS is part of the FET protein family, which are multifunctional DNA/RNA-binding proteins. Physiologically, these proteins are ubiquitously expressed and are usually localized in the nucleus of the cells. These proteins are imported to the nucleus by transportin-mediated mechanisms. Studies investigating the protein extracts from FTLD-FUS brain have shown an increase in the solubility of all FET proteins [56]. Thus, structural alterations in FET-family proteins, including arginine methylation and phosphorylation, might affect intracellular transport and thereby increase the chances of inclusion formation [35].

Because of the presence of FUS-positive inclusions and tau/TDP-43 negative inclusions, this condition was named the FTLD-FUS subgroup [35]. Recent studies have shown an association of mutations in the fused in sarcoma (FUS) gene with ALS [57]. However, it seems that FUS mutations are rarely associated with FTLD-FUS [58]. Also, imaging studies have shown that patterns of atrophy are less clear in patients with FUS mutations [59]. Interestingly, in contrast with ALS, FTLD-FUS presents co-accumulation of the proteins from the FET family EWS (Ewing sarcoma breakpoint region 1) and TAF15 (TATA-binding protein-associated factor 2N) and FUS inclusions [60]. It would therefore seem that the pathological mechanisms of FTLD-FUS may involve alterations in all FET

proteins. For this reason, in the near future, this subgroup may be renamed FTLD-FET.

Although many studies have shown a genetic association of mutations in the FET family with ALS, none of these demonstrated a genetic association of the mutations with FTLD-FUS, suggesting that FTLD-FUS is a sporadic disease [61].

CONCLUSION

We now know FTD to be a complex syndrome that can be divided into many subtypes based on clinical, genetic and pathological features. Over the past few years, a remarkable increase in our knowledge of the genetic, biochemical and pathological bases of FTD has helped us to better characterize the disorder. Animal models have also played a key role in dissecting the molecular and signalling pathways involved in FTD. Nevertheless, further investigation of the bases of FTD remains necessary in order to define specific biomarkers for the different subtypes of FTDL and, ultimately, to develop new and more effective therapeutic targets.

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CONFLICT OF INTEREST

The author(s) confirms that this chapter contents have no conflict of interest.

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CHAPTER 13

Charcot-Marie-Tooth Disease and Other Peripheral Neuropathies

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Abstract: Peripheral nerves connect the central nervous system with peripheral tissues in the body and are therefore crucial for all living animals to communicate with the environment. Due to the length of their axons, peripheral neurons are extremely vulnerable to insults. Inherited peripheral neuropathies comprise a large group of disorders characterized by progressive loss of axons or myelin that affect motor, sensory and/or autonomic nerves. Charcot-Marie-Tooth disease is the most common form of these inherited peripheral neuropathies. Peripheral nerves can also be damaged by a wide variety of stressors such as inflammation, infection, trauma, systemic disease, toxins/drugs and metabolic disturbances giving rise to several clinical subtypes of the disease. These disorders are referred to as acquired peripheral neuropathies. Ongoing research is focused on unraveling the pathogenic mechanisms underlying these debilitating diseases in order to find possible therapeutic strategies. So far, no drug therapy has been proven effective and patients have to rely on symptomatic treatments that are largely insufficient. Although there is no existing cure for peripheral neuropathies to date, some encouraging advances have been made which are also discussed in this chapter.

Keywords: Inherited Peripheral Neuropathies, Acquired peripheral neuropathies, Charcot-Marie-Tooth, Hereditary Motor and Sensory Neuropathy, Hereditary Motor Neuropathy, Hereditary Sensory and Autonomic Neuropathy, Myelination, nerves, Molecular mechanism, Therapy, Cytoskeleton, RNA metabolism, Membrane trafficking, Mitochondria, Immunity, Infections, Endocrinopathies, Systemic disease, Nutritional deficiencies, Chemotherapy.

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13.1. INTRODUCTION

The peripheral nervous system (PNS) consists of all parts of the nervous system that reside outside the central nervous system (CNS), formed by the brain and the spinal cord. The PNS can be subdivided into the visceral PNS also called the autonomic nervous system and into the somatic PNS [1].

Internal organs, blood vessels and glands are innervated by the visceral PNS and a distinction between motor and sensory visceral nerves can be made. The sensory axons collect information from organs, blood vessels and glands and send it to the CNS. The motor nerves control the contraction of smooth and cardiac muscles. These nerves also play a crucial role in the normal functioning of the glands. All nerves that connect the CNS to skin, joints and muscles are innervated by the somatic PNS. The motor nerves of the somatic PNS coordinate the skeletal muscle contraction and relaxation, while the sensory nerves and their axons receive information from skin, muscles and joints. The axons of the motor and sensory nerves are part of the PNS. On the other hand, the cell bodies of the motor neurons reside within the spinal cord while the cell bodies of the sensory neurons lie outside the CNS in the 'Dorsal Root Ganglia' (DRG) [1].

Unlike the CNS, peripheral nerves are not protected by the bone of the spine or the skull or by a tight blood - brain barrier. Only an incomplete blood-nerve barrier surrounds them. Therefore, peripheral nerves are more vulnerable to toxins and mechanical injuries. In this chapter, disorders of the PNS will be discussed that can affect both the visceral and the somatic nerves.

Neuropathies are characterized by a progressive length-dependent loss of nerve functioning and are a heterogeneous collection of disorders that occur as the PNS is damaged. Peripheral neuropathies are heterogeneous in etiology, pathology and clinical presentation that hamper their unifying classification and epidemiological studies. These disorders are classified as 'inherited peripheral neuropathies' (IPNs) when there is evidence for a genetic origin. On the other hand, inflammation, infectious diseases, trauma, ischemic insults, exposure to toxins/drugs and metabolic disturbances can also cause peripheral neuropathies. These disorders are categorized as 'acquired peripheral neuropathies' (APNs).

The first part of this chapter will focus on inherited peripheral neuropathies by discussing their classification and the clinical aspects of the different subtypes. We will also discuss recent advances in the understanding of potential underlying pathogenic mechanisms. Furthermore, we will highlight current treatment options for these patients together with experimental therapies that have shown beneficial effects in either slowing down or reversing the phenotype of transgenic mouse models of IPN. The second part will highlight the current knowledge regarding some of the more than 100 known acquired peripheral neuropathies. We will focus on Guillain-Barré syndrome and 'Human Immunodeficiency Virus' (HIV)-induced neuropathies as typical examples of neuropathies associated with immunity and infectious diseases, respectively. We will also discuss examples of neuropathies associated with endocrine disorders (diabetic neuropathy), with systemic disease (critical illness neuropathy), with nutritional deficiencies (alcohol-induced neuropathy) and with chemotherapy (vincristine-, taxane- and bortezomib-induced neuropathies).

13.2. INHERITED PERIPHERAL NEUROPATHIES

IPNs are characterized by progressive length-dependent degeneration of peripheral nerves and are further subdivided into three main groups. Patients suffer from 'Hereditary Motor and Sensory Neuropathies' (HMSN) also known as Charcot-Marie-Tooth disease (CMT) when both motor and sensory nerves are affected. 'Hereditary Motor Neuropathies' (HMN) are characterized by predominant motor deficits while 'Hereditary Sensory and Autonomic Neuropathies' (HSAN) are diagnosed when clinical signs involve sensory and/or autonomic nerves (Fig. **1**). This classification is merely based on clinical findings and was made before any underlying genetic cause was discovered [2-4]. The first causative genetic alteration identified as underlying cause of CMT type 1A, was the duplication of the 'Peripheral Myelin Protein 22' gene (PMP22), discovered about 20 years ago [5-7]. At present, gene defects in more than 50 genes are known to give rise to peripheral neuropathies and the identification of causative genes gives more insight into the underlying molecular mechanisms (for an overview of currently known causative genes: http://neuromuscular.wustl.edu/; http://www.molgen.ua.ac.be/CMTMutations). However, IPNs are characterized by genetic heterogeneity which means that one particular IPN phenotype can be

caused by gene defects in several genes. Together with the clinical heterogeneity – defined as gene mutations giving rise to different subtypes of IPN – the current classification of IPN becomes complex and insufficient.

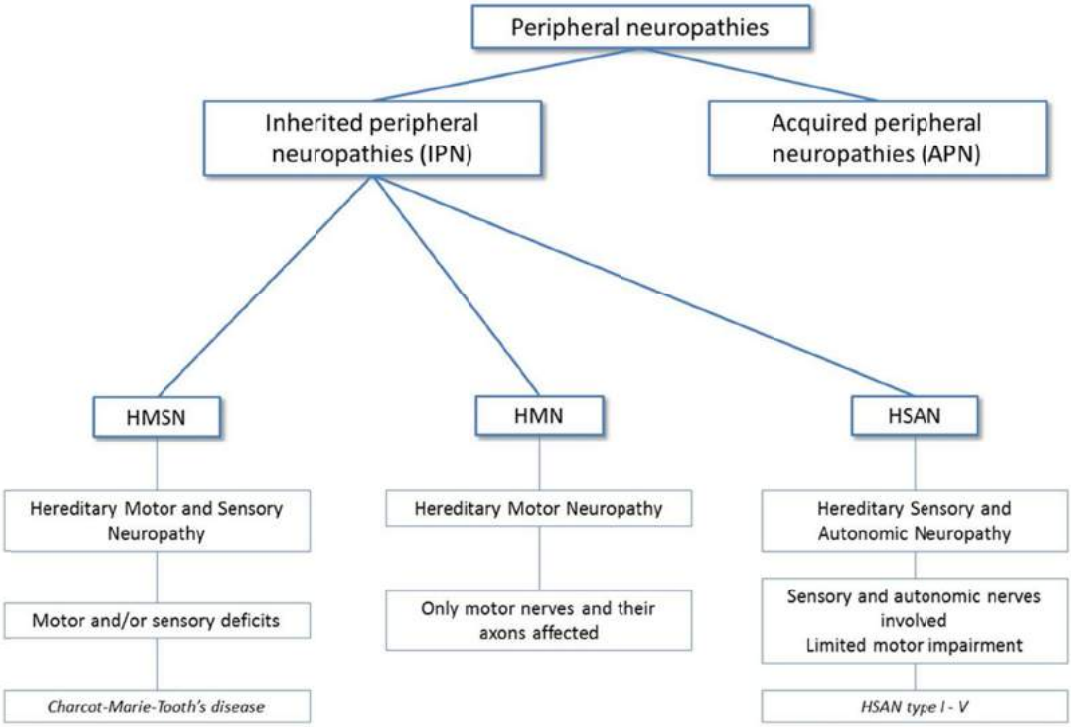

Figure 1: Classification of peripheral neuropathies. Classification of peripheral neuropathies based on family history or environmental factors. IPN, Inherited peripheral neuropathies; HMSN, Hereditary Motor and Sensory Neuropathies; CMT, Charcot-Marie-Tooth disease; HMN, Hereditary Motor Neuropathies; HSAN, Hereditary Sensory and Autonomic Neuropathies HSAN type I and familial dysautonomia (FD) are some examples of HSAN.

13.2.1. Hereditary Motor and Sensory Neuropathy and Hereditary Motor Neuropathy

The most common form of IPN is HMSN better known as CMT, with an estimated prevalence of 5-40 per 100,000 individuals [8]. The typical clinical signs of CMT do not only concern physical symptoms but the disease has also an important impact on the mental health of the patients [9]. Therefore, one of the outcome measures in clinical trials that test possible therapies for CMT is how the disease has an impact on the daily life of the patient. It is extensively studied in

adult patients although less well studied in children with CMT [10]. For instance, children aged from 5 to 18 years with varying types and severity of CMT exhibit lower physical, psychological and social well being than the general pediatric population [11]. In order to get better insights into the disease, it is essential to know the underlying genetic causes and to understand the pathogenic mechanism.

The first clear description of CMT was made almost simultaneously in 1886 by 3 neurologists: Jean Martin Charcot and Pierre Marie in France and Howard Henry Tooth in the United Kingdom [12]. The main characteristics of CMT are a combination of lower motor neuron and sensory defects [13]. The disease onset of CMT typically occurs during the first two decades of life and signs follow a slow "stocking-and-gloves" progression over time [8, 14]. Typical motor problems consist of distal muscle atrophy and weakness that first affect the intrinsic foot muscles and the peroneal muscles. Next, disease progresses up to the hands and forearms. The first manifestations become more severe and even scoliosis – a deformation of the spine – and skeletal deformities of feet and hands (including *pes cavus*, hammertoes and clawed hands) are observed. These symptoms cause steppage gait, difficulties in walking and running, muscle cramps and hand tremor [3, 15-17]. The sensory signs follow the same progression pattern but mainly affect feet and hands. CMT patients can suffer from negative sensory signs including loss of pain sensation and the absence of feeling vibration and touch. Furthermore, deep-tendon reflexes can be reduced or absent. Also positive sensory signs are observed and these include pain of the lower limbs and lumbar spine and paraesthesia, defined as a sensation of burning, prickling or itching of the skin without any obvious cause [18]. Depending on the severity of the disease, patients can become wheelchair-bound in later disease stages.

At present, CMT patients are subdivided into two main groups based on nerve conduction velocities (NCVs) of peripheral nerves. This provides a framework for both diagnostic and research purposes (Fig. **2**). CMT1 (or HMSNI) is primarily a demyelinating form of the disease characterized by decreased NCV (<38 m s^{-1}) [19]. A nerve biopsy of the *nervus suralis* from these patients shows myelin abnormalities with onion-bulb formation. This phenomenon appears when recurrent demyelination and re-myelination of peripheral nerves occurs as is seen in several human diseases like diabetic neuropathy and CMT [17]. The second

type of CMT (CMT2 or HMSNII) is predominantly an axonal form that can be distinguished from CMT1 by normal NCVs (> 38 m s^{-1}) but decreased amplitudes of the compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs). Motor and sensory nerve axons of CMT2 patients also display chronic axonal degeneration and regeneration [19].

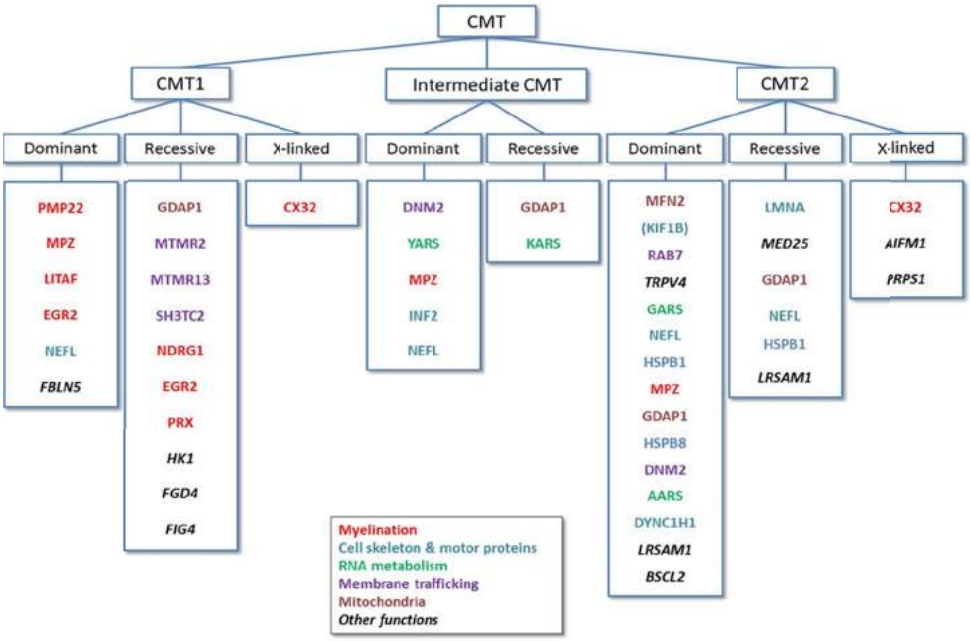

Figure 2: Classification of Charcot-Marie-Tooth disease. Classification of CMT based on NCV, mode of inheritance (dominant, recessive or X-linked) and the underlying gene defect. For each subtype of CMT the known causative genes are listed. CMT-causing genes were grouped together according to related cellular functioning and these genes are further discussed in detail. PMP22, peripheral myelin protein 22; MPZ, myelin protein zero; LITAF, lipopolysaccharide-induced tumor necrosis alpha-factor; EGR2, early growth response 2; NEFL, neurofilament light-chain; FBLN5, fibulin 5; KARS, lysyl-tRNA transferase; GDAP1, ganglioside-induced differentiation associated protein 1; MTMR2, myotubularin related protein 2; MTMR13, myotubularin related protein 13; SH3TC2, SH3 domain and tetratricopeptide repeats 2; NDRG1, N-myc downstream regulated 1; PRX, periaxin; HK1, hexokinase 1; FGD4, FYVE RhoGEF and PH domain containing 4; FIG4, polyphosphoinositide phosphatase; CX32/GBJ1, connexin-32/gap junction protein beta-1; DNM2, dynamin 2; YARS, tyrosine-tRNA transferase; INF2, inverted formin-2; MFN2, mitofusin 2; i, rab protein 7; TRPV4, transient receptor potential vanilloid 4; GARS, glycyl tRNA transferase; HSPB1, heat shock protein B1; HSPB8, heat shock protein B8; AARS, alanyl-tRNA transferase; DYNC1H1, dynein cytoplasmic 1 heavy chain 1; LRSAM1, leucine rich repeat and sterile alpha motif containing 1; BSCL2, Berardinelli-Seip congenital lipodystrophy 2; LMNA, lamin A/C; MED25, mediator complex subunit 25; AIFM1, apoptosis-inducing factor mitochondrion-associated 1; PRPS1, phosphoribosyl pyrophosphate synthetase 1.

If clear signs of both demyelination and axonal loss are observed, the disease is classified as an intermediate form of CMT and referred to as 'Dominant Intermediate' or 'Recessive Intermediate' CMT (DI- or RI-CMT) [17]. If only motor neurons and their axons are affected and thus the sensory signs are absent both clinically and electrophysiologically, the disease is referred to as 'distal Hereditary Motor Neuropathy' (distal HMN) [19]. CMT can be further subdivided according to the inheritance pattern and the underlying gene defect. Table **1** gives an overview of all CMT subtypes.

Table 1: Overview of different forms of CMT with the associated locus/gene(s) and OMIM reference number

	OMIM number	**Locus**	**Associated gene**	**Reference(s)**
CMT1 (dominant & demyelinating)				
CMT1A	118220	17p12	PMP22	[5, 6, 21]
CMT1B	118200	1q23.3	MPZ	[238, 239]
CMT1C	601098	16p13.13	LITAF	[240]
CMT1D	607678	10q21.3	EGR2	[241]
CMT1E	118300	17p12 1q23.3	PMP22 MPZ	[242]
CMT1F	607734	8p21.2	NEFL	[243]
CMT1	---	14q32.1	FBLN5	[244]
CMT4 (recessive & demyelinating)				
CMT4A	214400	8q13-q21.1	GDAP1	[101]
CMT4B1	601382	11q22	MTMR2	[84]
CMT4B2	604563	11p15.4	SBF2/MTMR13	[89]
CMT4C	601596	5q23	KIAA1985 (SH3TC2)	[245]
CMT4D	601455	8q24.3	NDRG1	[246]
CMT4E	605253	10q21.1-10q22.1	EGR2	[241]
CMT4F	145900	19q13.2	PRX	[247]
CMT4G	605285	10q23.2	HK1	[248]
CMT4H	609311	12p11.21	FGD4	[249]
CMTX (X-linked)				
CMTX1	302800	Xq13.1	GJB1/Cx32	[250]
CMTX2	302801	Xp22.2	Unknown	[251, 252]
CMTX3	302802	Xq26	Unknown	[253]
CMTX4	310490	Xq24-q26.1	AIFM1	[254]
CMTX5	311070	Xq22.3	PRPS1	[255]

Table 1: contd....

DI-CMT (dominant intermediate)				
CMT DIA	606483	10q24.1-q25.1	Unknown	[256]
CMT DIB	606482	19p13.2	DNM2	[257]
CMT DIC	608323	1p35.1	YARS	[74]
CMT DID	607791	1q23.3	MPZ	[258]
CMT DIE	614455	14q32.33	INF2	[71]
RI-CMT (recessive intermediate)				
CMT RIA	608340	8q21.11	GDAP1	[259]
CMT RIB	613641	16q23.1	KARS	[260]
CMT2 (dominant axonal)				
CMT 2A2	609260	1p36.22	MFN2	[96]
CMT 2B	600882	3q21.3	RAB7	[108]
CMT 2C	606071	12q24.11	TRPV4	[261]
CMT 2D	601472	7p14.3	GARS	[73]
CMT 2E	607684	8p21.2	NEFL	[57]
CMT 2F	606595	7q11.23	HSPB1	[262]
CMT 2G	608591	12q12-13.3	Unknown	[263]
CMT 2I	607677	1q23.3	MPZ	[264]
CMT 2J	607736	1q23.3	MPZ	[265]
CMT 2K	607831	8q21.1	GDAP1	[105]
CMT 2L	608673	12q24.3	HSPB8	[266, 267]
CMT 2M	606482	19p13.2	DNM2	[268]
CMT 2N	613287	16q22.1	AARS	[75]
CMT 2O	614228	14q32.31	DYNC1H1	[47]
CMT 2P	614436	9q33.3	LRSAM1	[269]
CMT 2	---	10p13-14	DHTKD1	[270]
CMT 2	---	11p11-11q13.3	BSCL2	[271]
CMT2 (recessive axonal)				
CMT 2B1	605588	1q22	LMNA	[272, 273]
CMT 2B2	605589	19q13.33	MED25	[274, 275]
CMT 2H	607731	8q13-q21.1	GDAP1	[276]
CMT 2K	607831	8q13-q21.1	GDAP1	[277]
CMT 2B5	---	8p21.2	NEFL	[278]
AR-CMT	608634	7q11.23	HSPB1	[279]
CMT 2P	614436	9q33.3	LRSAM1	[269]

All known forms of CMT are characterized by Mendelian inheritance and complete penetrance although the severity and the extent of the disease can vary between affected members of the same family [16]. Autosomal dominant forms account for 90% of all CMT cases reported in Northern Europe, the United Kingdom (UK) and the United States (US), but X-linked inheritance is also observed [17]. Approximately 80% of the cases are classified as CMT1, while 20% of patients are categorized as CMT2 [20]. Other forms of CMT are considered to be rare. CMT is known as a genetically heterogeneous disorder and thus far, over 50 different genes have been associated with CMT (Fig. **2** and Table **1**) [17, 18].

13.2.2. Molecular Mechanisms Causing Charcot-Marie-Tooth disease and Hereditary Motor Neuropathy

13.2.2.1. Gene Mutations Affecting Myelination of Peripheral Nerves

Mutations in a number of genes that play a role in myelination of peripheral nerves by Schwann cells are found to cause different subtypes of CMT. These genes include 'Peripheral Myelin Protein 22' (PMP22), 'Myelin Protein Zero' (MPZ), 'Early Growth Response 2' (EGR2), 'N-myc Down-Regulated Gene-1' (NDRG1), 'Periaxin' (PRX) and 'Connexin-32' (Cx32 or GBJ1). We will discuss their normal function as well as possible pathogenic mechanisms.

In 50% of all CMT cases the underlying genetic defect is an intra-chromosomal duplication of a region containing the PMP22 gene [5, 6]. However, not only duplications but also point mutations in PMP22 have been reported to cause autosomal dominant CMT1A [21]. PMP22 is mainly expressed in Schwann cells and plays a role in formation and maintenance of the myelin sheets [22]. More insight into the pathogenic mechanism came from two CMT1A mouse models harboring spontaneous heterozygous mutations in endogenous Pmp22: Trembler (Tr) mice expressing p.G150D Pmp22 and Trembler J (Tr-J) mice with a p.L16P mutation in Pmp22 [23, 24]. Malfunctioning of myelinating Schwann cells can have a deleterious effect on peripheral nerve axons. In Tr mice, demyelination decreased neurofilament phosphorylation but increased neurofilament density, leading to a decrease of both slow axonal transport and axon diameter [25]. Moreover, demyelination also affected the microtubule cytoskeleton [26]. The

microtubules in peripheral axons of Tr mice appeared unstable and the composition and phosphorylation of microtubule-associated proteins was altered [26]. In addition, Schwann cells of Tr-J mice displayed an up-regulated endosomal-lysosomal pathway in combination with the presence of Pmp22-containing aggregates [27, 28].

PMP22 is known to interact with MPZ that mediates membrane adhesion in myelin sheaths and this interaction is also required for proper myelination [29]. Interestingly, mutations in MPZ can not only cause demyelinating CMT (CMT1B), but can also be the underlying gene defect of axonal CMT (CMT2I and CMT2J) or DI-CMT [30]. It is proposed that a change in PMP22 expression levels leads to a disturbance in the balance of PMP22/MPZ levels causing myelin abnormalities [31].

One of the factors regulating the expression of PMP22 and MPZ is EGR2. This transcription factor is important for myelin development and maintenance by Schwann cells of the peripheral nervous system [32]. Moreover, EGR2 controls the expression of a large number of myelin-associated genes [33]. Mutations in EGR2 can result in dominant or recessive demyelinating CMT (CMT1D and CMT4E, respectively. Dominant mutations in EGR2 negatively influence the activation of myelin-associated genes like PMP22 and MPZ in Schwann cells by wild-type EGR2 [34].

Autosomal recessive mutations in NDRG1 are the underlying cause of CMT4D or HMSN-Lom (HMSN-L). NDRG1 is highly expressed in the cytoplasm of Schwann cells but is not detected in motor and sensory neurons [35]. The exact physiological function of NDRG1 is not yet fully understood. NDRG1 was linked to a number of cellular processes. For instance, through identification of its interaction partners, apolipidproteins APO-AI and APO-AII, NDRG1 would be implicated in cellular trafficking of lipids, reverse cholesterol transport but also endosomal recycling [36, 37]. NDRG1 is present at early stages of myelin degradation but is depleted at the end of this process [35]. Involvement of NDRG1 in myelin maintenance is further supported by the phenotype of Ndrg1 deficient mice. These mice exhibit normal myelination of sciatic nerves shortly

after birth, while later in life demyelination and degeneration of peripheral nerves becomes apparent [38, 39]. Despite myelin abnormalities, Ndrg1-deficient mice do not fully replicate human CMT4D suggesting that the pathogenic mechanism of mutant NDRG1 is not simply a 'loss-of-function'.

Another protein involved in proper myelination of the peripheral nerves is periaxin (encoded by PRX). This protein is expressed at the surface of Schwann cells and is part of the dystroglycan complex through its interaction with 'Dystrophin-Related Protein 2' (DRP2) [40]. The PRX-DRP2 interaction is responsible for linking the basal lamina of the extracellular matrix to the cytoskeleton of Schwann cells [40, 41]. Mutations in PRX give rise to autosomal recessive demyelinating CMT (CMT4F) and Déjèrine-Sottas neuropathy, a subtype of CMT also named HMSN type III. It is proposed that mutations generate a truncated protein leading to a 'loss-of-function' mechanism with disruption of the PRX-DRP2 complex [42].

Closely packed pairs of transmembrane channels, the connexons, form gap junctions consisting of different connexins. Connexins mediate and regulate the exchange of ions and small metabolites between adjacent cells. More than 400 different mutations in CX32, also named 'Gap-Junction Beta-1' (GJB1), can be the genetic cause of both demyelinating and axonal X-linked forms of CMT (CMTX1). The disease course in affected males can be severe while heterozygous females are usually less affected or are only carriers [43]. Different types of mutations have been described (nonsense, missense, frame-shift or deletions) all leading to a 'loss-of-function' of CX32. However, the pathogenic mechanism can be different. Some mutations lead to non-functional channels or abolish the CX32 expression, while others cause retention of CX32 in the endoplasmic reticulum (ER) or Golgi apparatus or an abnormal cellular distribution of CX32 thereby altering the biophysical functions or the trafficking of the protein within the cell [41, 43]. Cx32-deficient mice closely mimic the CMTX1 phenotype seen in humans [44, 45]. Interestingly, this phenotype could be rescued by the Schwann cell-specific expression of Cx32, indicating a Schwann cell based pathogenic mechanism [46].

13.2.2.2. Gene Mutations Affecting Cytoskeletal Structures and Proper Motor Protein Functioning

Not only genes composing the cytoskeleton or nuclear membrane are mutated in CMT, but also mutations in genes encoding motor proteins and molecular chaperones that bind these cytoskeletal components are the underlying cause of HMSN. Here we discuss both normal function and possible pathogenic mechanisms regarding 'Dynein, Cytoplasmic 1, Heavy chain 1' (DYNC1H1), Dynactin subunit-1 (DCTN1), 'Lamin A/C' (LMNA), 'Neurofilament Light-chain' (NEFL), 'small Heat Shock Proteins B1/B3/B8' (HSPB1/HSPB3/HSPB8) and 'Inverted Formin 2' (INF2).

Mutations in DYNC1H1 lead to axonal CMT, while mutations in the $p150^{glued}$ subunit of dynactin give rise to distal HMN [47, 48]. The dynactin-complex is required for microtubule-based retrograde axonal transport of vesicles and organelles, mediated by dynein [49]. Binding assays show a reduced binding of mutant dynactin-1 to microtubules leading to axonal transport deficits [48].

Furthermore, genes composing the cytoskeleton or the nuclear membrane have also been implicated in CMT. Lamin A/C (LMNA) belongs to the family of lamins constituting the nuclear lamina, important for the structure of the nuclear envelope and these proteins also interact with chromatin [50, 51]. Autosomal recessive mutations give rise to CMT2B1 in humans [52]. A knock-in mouse model homozygous for p.R298C LMNA does not develop a peripheral neuropathy but showed downregulation of LMNA and upregulation of Pmp22 specifically in sciatic nerve [53]. In contrast, targeted disruption of Lmna in mice caused a reduction in axon density and an increase in the axon diameter and the number of non-myelinated axons [54]. As a consequence, these mice can serve as a model for mutant LMNA induced CMT2 [54]. Interestingly, lamin B2, an intermediate filament protein, is locally translated in axons while it is also associated with the nuclear membrane [55]. Inhibition of the local axonal translation of lamin B2 leads to axonal degeneration and disruption of mitochondria as lamin B2 is also associated with mitochondria [55].

Mutations in another component of the intermediate filaments, NEFL, can lead to the development of demyelinating, axonal and intermediate forms of CMT

depending on the underlying mutations [56, 57]. Studies involving CMT2-causing mutations in NEFL showed disruption of neurofilament assembly and neurofilament aggregation but also mitochondrial dysfunction has been reported [58, 59]. Co-expression of wild-type HSPB1 with mutant NEFL leads to less pronounced aggregation and decreases mutant NEFL-induced loss of the motor neuron survival [58].

Mutations in HSPB1 lead to the development of CMT2 or distal HMN. CMT2-causing mutant HSPB1 also disrupts NEFL assembly and leads to the formation of NEFL- and HSPB1-containing aggregates [60]. HSPB1, primarily known as a molecular chaperone, belongs to the family of small heat shock proteins (small HSPs) but also interacts with components of the cytoskeleton including subunits of microtubules, intermediate filaments and microfilaments [61]. Some mutant HSPB1s displayed increased binding to tubulin and these microtubules showed increased resistance to cold- and nocodazole-induced depolymerization in the presence of mutant HSPB1 [62, 63]. Moreover, a transgenic mouse model for CMT2 and distal HMN over-expressing mutant HSPB1 showed decreased acetylated tubulin levels in peripheral nerves together with axonal transport defects of mitochondria [64]. Furthermore, also dominant mutations in other family members of the HSPs, including HSPB3 and HSPB8, lead to the development of CMT or distal HMN [65, 66]. Missense mutations in HSPB8 cause neurite degeneration in primary motor neuron culture although no signs of apoptosis were present. Fibroblasts from distal HMN patients carrying HSPB8 mutations show aggregation of mutant HSPB8 together with a reduced mitochondrial membrane potential [67, 68]. Interestingly, overexpression of mutant HSPB8 in a motorneuron-like cell line impaired autophagy. Autophagosomes co-localized with protein aggregates in this cell line but failed to fuse with the lysosomes. These defects in autophagy were also observed in fibroblasts from distal HMN patients with mutations in HSPB8 [69].

INF2 is involved in actin dynamics as it mediates actin filament assembly by accelerating actin nucleation and elongation at the barbed end through its ‘Formin Homology 2’ (FH2) domain. The C-terminal region of INF2 is required for depolymerization of actin filaments [70]. An autosomal dominant intermediate form of CMT can also be associated with renal diseases like focal segmental

glomerulosclerosis and this particular phenotype is caused by mutations in INF2 [71]. Interestingly, INF2 is strongly expressed in podocytes and Schwann cells and interacts with an actin-regulating Rho-GTPase, CDC42, and myelin and lymphocyte protein (MAL) [72]. The aforementioned proteins are all involved in myelin formation and maintenance. Mutant INF2 causes disruption of cytoskeletal organization, enhanced binding with MAL and mislocalization of INF, MAL and CDC42 [71].

13.2.2.3. Gene Mutations Altering RNA Metabolism

Mutations in genes encoding 'Aminoacyl-tRNA Synthetases' (ARS), including 'Tyrosine-tRNA Synthetase' (YARS), 'Lysine-tRNA Synthetase' (KARS), 'Alanine-tRNA Synthetase' (AARS) and 'Glycyl-tRNA Synthetase' (GARS), cause autosomal dominant forms of CMT including DI-CMT and CMT2 but also RI-CMT [73-75].

These enzymes catalyze the binding of tRNA molecules with their cognate amino acids, a process crucial for proper RNA translation into proteins [76]. In addition, mutations in the mitochondrial isoform of 'Aspartyl-tRNA Synthetase' (DARS) lead to an axonal neuropathy in some patients [77]. Some mutations in GARS and YARS affect the enzymatic activity although it is still under debate whether this is part of the pathogenic mechanism since it is not the case for all mutations [78]. An N-Ethyl-N-nitroso-ureum (ENU)-induced mouse model for CMT2 carrying an endogenous mutation in Gars did not show a reduced enzymatic activity of Gars [78]. One study suggested that the underlying defect is a dose-dependent toxic 'gain-of-function' since overexpression of wild-type GARS could not rescue the dominant neuropathy in two mutant Gars-induced mouse models [79]. It has been shown that endogenous GARS localizes in granules present in the nucleus. In addition, smaller GARS-containing granules was also found in the cell body and in neurites of mouse motor neuron cell lines [80]. Interestingly, some mutations in GARS and YARS lead to different protein localization compared to wild-type protein [74, 80].

13.2.2.4. Gene Mutations Affecting Membrane Trafficking

'Lipopolysaccharide-Induced Tumor Necrosis Factor-Alpha Factor' (LITAF) - also named 'Small Integral Membrane Protein of Lysosome/late Endosome' (SIMPLE) -

is a potential E3 ubiquitin ligase of the RING finger motif-containing E3 subfamily. Patients carrying dominant mutations in LITAF develop demyelinating CMT (CMT1C) [81]. A Schwann cell based pathogenic mechanism was proposed since LITAF is highly expressed in Schwann cells although the proper function of LITAF remains elusive [41]. LITAF has been detected in the plasma membrane, Golgi apparatus and lysosomal membrane [41]. LITAF interacts with 'Neural Precursor Cell Expressed, Developmentally Down-regulated 4' (NEDD4), an E3 ubiquitin ligase that is responsible for the regulation of lysosomal degradation of membrane proteins by mono-ubiquitination. Furthermore, LITAF interacts with 'Tumor Susceptibility Gene 101' (TSG101) that acts downstream of NEDD4 and is involved in lysosomal sorting of ubiquitinated proteins into multivesicular bodies and subsequent degradation [82]. How mutations lead to the development of CMT1C is currently unclear but it has been shown that some of the mutations do not affect the interaction with NEDD4 and TSG101, nor lead to an altered subcellular localization [82]. However, recently it was shown that CMT-causing mutations in SIMPLE impair the signaling and trafficking of ErbB (epidermal growth factor receptor) by a dominant-negative mechanism resulting in a prolonged ERK1/2 signaling downstream of the ErbB receptors [83].

Autosomal recessive mutations in the genes encoding 'Myotubularin-related Proteins 2 and 13' (MTMR2 and MTMR13) lead to phenotypically similar subtypes of CMT (CMT4B1 and CMT4B2, respectively) [84]. The substrates of MTMR2 and MTMR13 are phosphatidyl-inositol 3-phosphate (PI-3-P) en phosphatidyl-inositol 3,5-bisphosphate (PI-3,5-P2), respectively and these phospho-inositides are regulators of endocytosis, membrane homeostasis and vesicular transport [41]. MTMR2 is the enzymatic active family member and mutations lead to a reduced phosphatase activity, while wild-type MTMR13 is functionally inactive [85]. Mtmr2-null mice develop a progressive neuropathy and Schwann cell-specific deletion also results in myelin abnormalities. These findings indicate a Schwann-cell based mechanism underlying the disease and are in line with the human phenotype [86]. Another binding partner of MTMR2 is NEFL which, as described above, also causes CMT [87, 88].

SH3TC2/KIAA1985 encodes the 'SH3 Domain and Tetratricopeptide Repeat-containing Protein 2' which is highly expressed in brain, spinal cord and

peripheral nerve Schwann cells. This protein is anchored to the plasma membrane and also localizes to the perinuclear endocytotic compartment, more specifically in early endosomes, late endosomes and clathrin-coated vesicles close to the trans-Golgi network. Autosomal mutations have been reported to be the underlying gene defect of CMT4C [89]. Missense mutations affect the localization in endosomes and the plasma membrane, while nonsense mutations do not alter the intracellular distribution. Neuropathy-causing mutations disrupting the interaction with the small guanosine triphosphatase Rab11 link SH3TC2 to endosomal recycling of internalized membranes and receptors back to the plasma membrane [90].

The small GTPase 'Ras-related Protein Rab-7' (RAB7) plays an important role in the endocytotic trafficking by controlling the conversion of late endosomes to lysosomes [91]. It is also involved in the long-distance retrograde transport of endosomal vesicles containing several neurotrophins [92]. Mutant RAB7 disrupted the neurite outgrowth in neuroblastoma cell lines and rat primary neurons [93]. Furthermore, mutations in RAB7 impaired the GTPase activity and increased the nucleotide exchange rate, but slowed down the hydrolysis of GTP [93-95]. Interestingly, HSAN type V and HSAN type IV are caused by mutation in β-nerve growth factor (β-NGF) and its receptor TrkA which are transported by the RAB7-mediated endocytotic sorting.

13.2.2.5. Gene Mutations Affecting Mitochondrial Function

Mutations in the genes encoding 'Mitofusin-2' (MFN2) and 'Ganglioside-induced Differentiation-associated Protein-1' (GDAP1) can give rise to distinct subtypes of CMT. Moreover, dominant mutations in MFN2 are the most common cause of CMT2 [96].

MFN2 is involved in mitochondrial fusion and regulation of mitochondrial oxidative function and also interacts with the Miro/Milton complex that serves as an adaptor for mitochondria to link them with motor proteins [97]. Mutant MFN2 disrupts axonal transport of mitochondria in cultured dorsal root ganglion (DRG) neurons that overexpress mutant MFN2 [98]. Furthermore, *nervus suralis* biopsies of patients carrying mutations in MFN2 are characterized by an accumulation and degeneration of mitochondria [98, 99]. Fibroblasts from patients showed a

mitochondrial energy coupling defect with a reduced mitochondrial membrane potential, while ATP production was unaffected [100].

GDAP1 also plays a role in mitochondrial fusion and fission. Recessive mutations lead to demyelinating or intermediate CMT (CMT4A and RI-CMT), while dominant mutation give rise to CMT2K [101-104]. Dominant mutations lead to a decrease in mitochondrial respiratory chain complex I activity and decreased ATP production in patient-derived fibroblasts. Defects in mitochondrial morphology were also observed [105].

13.3. HEREDITARY SENSORY AND AUTONOMIC NEUROPATHIES

'Hereditary Sensory Neuropathies' (HSN), also known as 'Hereditary Sensory and Autonomic Neuropathies' (HSAN) are a group of clinically and genetically heterogeneous disorders that predominantly affect the axons of sensory and autonomic nerves. However, varying motor involvement can be present making the distinction between CMT and HSAN sometimes difficult [106]. As an example, a large American family displaying features of an inherited axonal neuropathy was classified as CMT2B with the locus assigned to the gene encoding RAB7 [107, 108]. However, the presence of ulcerations and amputations as a consequence of sensory deficits argues in favor of the classification as HSAN type I [109].

Typical symptoms are altered pain and temperature sensation leading to chronic skin ulcerations on feet and hands [110]. Severe complications consist of osteomyelitis, spontaneous fractures, neuropathic arthropathy and the necessity of amputation of the affected limb [110, 111]. Autonomic deficits are variable, often unique to a HSAN subtype and can include altered sweating (hyperhidrosis or anhidrosis), cardiovascular dysregulation, postural hypotension and gastrointestinal motility problems [112].

The first classification of HSAN - made by Dyck and colleagues before the first gene defects were discovered - was based on the age of onset, mode of inheritance and the degree of sensory and/or autonomic involvement, resulting in five main subtypes: HSAN type I to V (Fig. **3**) [2]. HSAN type I can be distinguished from the other subtypes by its autosomal dominant inheritance pattern, a juvenile or

adulthood disease onset and minor autonomic deficits [113]. In some cases, motor symptoms are apparent making this form very similar to CMT as described earlier. The other subtypes (HSAN type II – V) are inherited in an autosomal recessive manner and typically show congenital or early-in-life disease onset [113]. HSAN type II, also referred to as congenital sensory neuropathy, has a very low worldwide prevalence [113]. There is predominantly sensory involvement with loss of pain, temperature and touch sensation and typical complications like auto-amputations, ulcerations and loss of deep tendon reflexes. The earliest problems are due to autonomic disturbances like gastro-esophageal reflux, feeding problems and apnea [110, 113]. HSAN type III, also known as 'Familial Dysautonomia' (FD) or Riley-Day syndrome is predominantly autonomic and extremely rare in most populations. However, it appears very frequently in Ashkenazi Jews where 1 in 30 people are FD carriers and an estimated disease incidence of 1 in 3600 births [114, 115]. Patients typically suffer from a 'dysautonomic crisis' that is characterized by episodes of nausea and vomiting, cardiovascular dysregulation such as hypertension and tachycardia, increase in gastrointestinal secretions and negative personality changes. These signs appear in about 40% of the FD patients and can occur with different intervals [113]. Congenital insensitivity to pain with anhidrosis (CIPA) is another description for HSAN type IV with a true sensory and autonomic involvement and with several hundreds of reported cases [113]. HSAN type V is phenotypically very similar to HSAN type IV and differs as patients suffering from the latter displays anhidrosis and mental retardation [110, 116]. Recently, another subtype has been described, HSAN type VI, with symptoms very similar to familial dysautonomia although the disease course is more severe [112].

Common to all subtypes of HSAN is the complete penetrance although the disease expression can vary considerably. Moreover, the lack of axon flare in response to intradermal histamine injection is a typical feature in most HSAN patients and is therefore used as a diagnostic tool [112, 113]. During this test, a dosage of histamine phosphate is injected intradermally which leads in healthy patients to a bright red histamine flare due to capillary vasodilatation. This response is due to an axon reflex within dermal nerves and is dependent on unmyelinated C-fibers. However, in some HSAN type II and HSAN type V patients having largely

preserved or little reduction in the unmyelinated C-fibers, this test appears normal [113]. In general, diagnosis of HSAN is based on clinical features and the degree of sensory and autonomic malfunctioning.

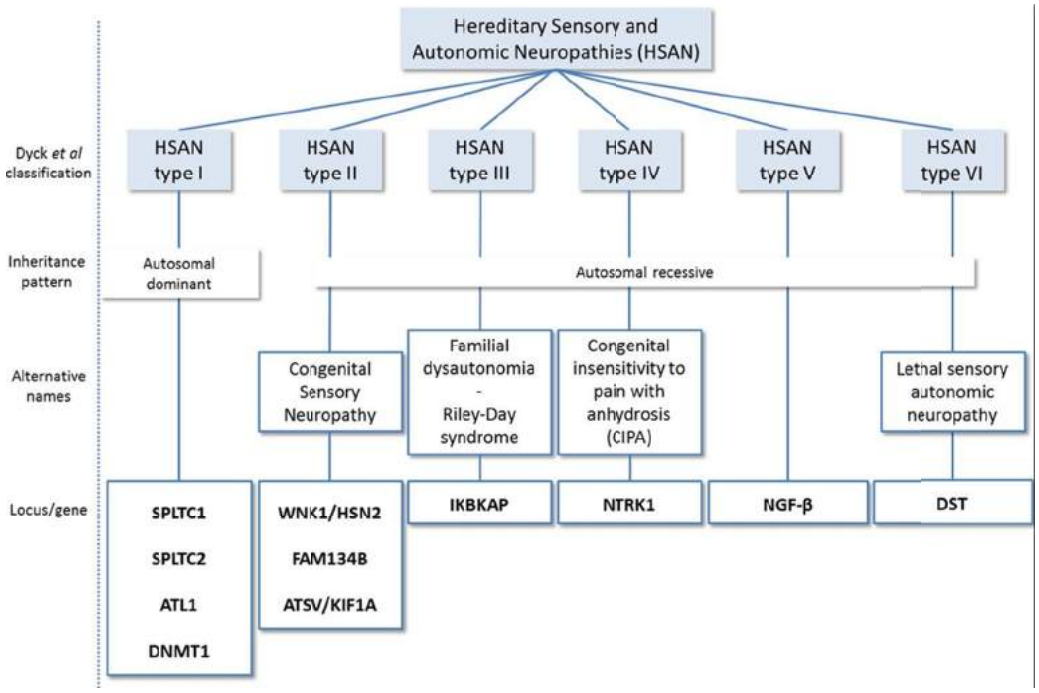

Figure 3: Classification of HSAN. The classification described is based on the age of onset, mode of inheritance and the degree of sensory and/or autonomic involvement resulting in five subtypes: HSAN type I – V. HSAN was described and found to be caused by mutations in DST (dystonin). Serine PalmitoylTransferase Long Chain subunit 1 (SPTLC1); Serine PalmitoylTransferase Long Chain subunit 2 (SPTLC2); Atlastine-1 (ATL1); DNA-metyltransferase 1 (DNMT1); serine/threonine-protein kinase (WNK1); Hereditary Sensory Neuropathy 2 (HSN2); Axonal Transport of Synaptic Vesicles (ATSV); IκB-kinase complex-associated protein (IKBKAP); Neurotrophic Tyrosine Kinase Receptor type 1 (NTRK1); Neurite Growth Factor β (NGF-β); dystonin (DST).

13.3.1. Molecular Mechanisms Causing Hereditary Sensory and Autonomic Neuropathies

Mutations in several genes have already been identified as the underlying cause of different HSAN subtypes although not all cases can be explained by these gene defects. Moreover, proteins encoded by HSAN-associated genes exert a wide variety of cellular functions including sphingolipid biosynthesis, DNA

methylation, motor proteins and transcriptional elongation. This diversity hampers the search for a general pathogenic mechanism underlying the different HSAN subtypes. An overview of the known causative genes is shown in Fig. **3**.

Heterozygous missense mutations in SPTLC1 and SPTLC2 (encoding 'Serine Palmitoyltransferase Long Chain' subunit 1 and 2) cause HSAN type IA and HSAN type IC and these patients are phenotypically indistinguishable [117-120]. SPTLC1 and SPTLC2 encode subunits of the 'Serine Palmitoyltransferase' (SPT) which catalyzes the first and rate-limited step in the biosynthesis of sphingolipids by condensation of L-serine with palmitoyltransferase-CoA [121]. No mutations in another subunit of SPT (SPTLC3) have been identified [119]. So far, five mutations in SPTLC1 have been reported and all lead to a reduced *in vitro* enzymatic SPT activity, although not affecting the *de novo* sphingolipid biosynthesis and cellular sphingolipid content [122]. Mutant SPTLC1 or SPTLC2 lead to a shift in substrate specificity from L-serine to L-alanine or L-glycine generating two toxic deoxysphingolipids. These metabolites accumulate in the cell due to the lack of a hydroxyl group and the subsequent failure of degeneration [123, 124]. Elevated levels of these toxic deoxysphingolipids were also detected in plasma of HSAN type I patients and in mice expressing mutant SPTLC1. These data suggest that reversing the change in substrate specificity of SPT could be a possible therapeutic strategy.

Mutations in 'Atlastin-1' (ATL1) are a frequent cause of 'Hereditary Spastic Paraplegia' (HSP) [125]. HSP is characterized by degeneration of upper motor neurons, muscle weakness and spasticity of the lower limbs [126, 127]. In some cases, mutations in ATL1 are also associated with a sensory neuropathy (HSAN type ID). The HSAN causing-mutations are scattered throughout ATL1 and no clear correlation between the localization of the mutations and the resulting phenotype (HSAN type I or HSP) could been found [119]. ATL1, a dynamin-like GTPase, is mostly localized to vesicular tubular complexes and *cis*-Golgi cisternae and mutations are known to interfere with vesicle trafficking in ER/Golgi and in Golgi morphogenesis [128, 129]. *In vitro* studies showed that mutations in ATL1 have a dominant-negative effect on the GTPase activity but further research is needed to elaborate the pathogenic mechanism(s) [130].

HSAN type IE is often associated with early-onset dementia and sensorineuronal hearing loss resulting from mutations in the DNA-methyltransferase 1 (encoded by DNMT1) [131]. Missense mutations in DNMT1 are localized in the DNA-targeting sequence domain responsible for the chromatin binding during the S-phase and the maintenance of this binding during G2 and M phase of mitosis. Mutant DNMT1 has been shown to possess decreased enzymatic activity but also to be misfolded causing the protein to be retained in the cytoplasm, premature degradation and therefore failure of binding to the heterochromatin during M phase and after the S phase. In addition, genomic DNA from patients showed global hypomethylation although local hypermethylation was also present [131].

The first causative mutations for HSAN type II were discovered in HSN2, a gene consisting of one exon with a neuronal-specific expression especially in DRG and sciatic nerves [132]. Later on, it was discovered that this gene resides in the intron 8 of WNK1 and is expressed as a tissue-specific isoform of the serine/threonine-protein kinase WNK1 [133]. WNK1 belongs to the family of serine/threonine kinases that regulate the sodium, potassium and chloride homeostasis and is also able to downregulate TRPV4 expression, an ion channel responsible for temperature, osmo- and mechanosensation [134, 135]. Interestingly, patients heterozygous for WNK1 mutations display an increased cold/heat and pain response [136]. A yeast-two-hybrid screen identified kinesin-like protein, KIF1A, as an interaction partner of WNK1 [133]. Additional genome-wide homozygosity mapping and subsequent sequencing revealed that truncations in KIF1A are also responsible for HSAN type IIC in humans [137]. KIF1A, also known as 'Axonal Transport of Synaptic Vesicles' (ATSV) is a molecular motor protein responsible for the anterograde transport of synaptic vesicles. The most common mutation occurs in an alternative splice variant of KIF1A expressed specifically in neurons [137]. The pathogenic mechanism of KIF1A mutations is poorly understood. It was suggested that KIF1A could be responsible for the transport of WNK1 since both proteins interact. However, knock down of KIF1A did not affect the subcellular localization of WNK1 in cultured DRG neurons. Conversely, it was suggested that WNK1 plays a role in the unloading of KIF1A transported vesicles as the expression of both proteins seems to be enriched at the axon tip [137].

Mutations in FAM134B, part of a family of three genes (FAM134A, FAM134B, FAM134C) give rise to HSAN type IIB. At present, not much is known about the normal function of the gene products or the pathogenic mechanism behind these mutations [138, 139]. FAM134B is highly expressed in sensory and autonomic ganglia and is localized to the *cis*-Golgi. Moreover, knockdown of FAM134B induced apoptosis in primary dorsal root ganglion neurons and caused structural changes to the Golgi apparatus [139].

HSAN type III, also known as 'Familial Dysautonomia' (FD) or Riley-Day syndrome is in most cases caused by mutations in IKBKAP ('IκB Kinase Complex-associated Protein' or 'Elongator Complex Protein 1', ELP1) which plays a role in the transcriptional elongation [140, 141]. The most common mutation, accounting for 99.5% of all cases, causes a splice defect at the 5' splice site of intron 20 leading to the skipping of exon 20 although varying levels of wild-type IKAP are still expressed. This splice defect occurs tissue-specific since in lymphoblasts and fibroblasts of affected patients the wild-type RNA is primarily found, while the mutant RNA is more abundant in brain tissue [141]. As this splice mutation explains the majority of all FD cases, it forms the basis for rapid screening of possible carriers in the population. Moreover, the varying expression levels of wild-type IKAP argue in favor of increasing the expression levels of IKAP as a therapeutic strategy (see below) [141]. Recently, the first mouse model for FD was created and it was shown that varying mutant IKAP expression levels can modulate the disease severity of FD [142].

Missense, nonsense, frame-shift and splice-site mutations in NTRK1 ('Neurotrophic Tyrosine Kinase Receptor, type 1'), encoding the high-affinity nerve growth receptor TrkA have been reported as the cause of HSAN type IV. The mutations are scattered along TrkA and the corresponding pathogenic mechanisms widely differ, but all point to defects in molecular signaling [110]. Interestingly, one of the TrkA ligands, β-NGF, which plays a role in development and function of nociceptive and sympathetic sensory neurons is also involved in HSAN [143]. Mutations in β-NGF lead to HSAN type V in humans. Moreover, mutations in TrkA have also been reported in a family diagnosed with HSAN type V [144].

Recently, a new subtype of HSAN was reported [112]. Within a large consanguineous family from Ashkenazi Jewish origin three children displayed clinical features of familial dysautonomia although the disease course and outcome was more severe [112]. Dysautonomic symptoms including absent tearing, blotching, feeding difficulties and absent deep tendon reflexes were present. Additional contractures were also described together with severe psychomotor retardation and early death. This new form of HSAN is now referred to as lethal autonomic sensory neuropathy. Homozygosity mapping revealed that the disease was caused by a mutation in the DST gene which encodes a cytoskeletal linker protein dystonin. Dystonin belongs to the family of plakin proteins, known for the linkage of cytoskeletal proteins (intermediate filaments, actin microfilaments and microtubuli) [145]. The frame-shift mutation leads to the generation of an unstable mRNA transcript and is predicted to cause the loss of part of the C-terminus of the dystonin protein [112]. Further research is needed to explore how this frame-shift mutation leads to neurodegeneration of the peripheral nervous system.

13.3.2. Therapeutic Interventions to Treat Inherited Peripheral Neuropathies

To date, treatment of inherited peripheral neuropathies only consists of supportive measures and is in most cases insufficient to ease all the symptoms. CMT patients can rely on rehabilitation, orthics, symptomatic drug therapy of pain and the surgical corrections of foot and hand deformities [145-147]. Patients suffering from HSAN need supportive treatment of the ulcerations, osteomyelitis and amputations. Genetic testing is applied in case of familial dysautonomia since 99.5% of all cases are caused by a splice-site mutation in IKBKAP. This FD carrier screening has a great influence on the number of patients suffering from FD as a large number of affected pregnancies are avoided or terminated in early phase [148].

Most research and clinical studies are focused on CMT1A pathology caused by PMP22 gene duplication. The most commonly used models are transgenic animals, either mice or rats, overexpressing PMP22. These animal models recapitulate peripheral demyelination, axonal loss and subsequent muscle atrophy as seen in CMT1A patients carrying the PMP22 mutation [149, 150].

Progesterone promotes myelination in the peripheral nervous system and stimulates the PMP22 and MPZ expression in Schwann cell cultures [151-153]. Therefore, the progesterone receptor could be a potential therapeutic target in CMT1A. Treatment of PMP22 overexpressing rats with a progesterone antagonist (onapristone) leads to a clinical and neuropathological improvement of the CMT1A phenotype and had a protective effect on axonal loss [154, 155]. However, onapristone is known to be too toxic to treat humans [156]. As a consequence, the development of new progesterone antagonists with less toxic side effects in humans should be considered.

Ascorbic acid is well known for its effects on the formation of the collagen- and laminin-containing extracellular matrix in Schwann cell/DRG co-cultures and promotes Schwann cell-dependent myelination [157-160]. *In vivo* experiments confirmed that ascorbic acid promotes myelination of the PNS. On the other hand, mice in which the sodium-dependent vitamin C transporter-2 is disrupted develops sensorimotor impairment due to hypo-myelination and collagen formation defects in the peripheral nerves by lowered uptake of ascorbic acid [161]. Administration of ascorbic acid to PMP22-overexpressing mice partially corrects their motor defects through re-myelination of peripheral axons combined with a decrease in the PMP22 expression levels [162]. Despite these promising results, three clinical trials have failed to prove beneficial effects of ascorbic acid supplementation to CMT1A patients [163-165]. Recently, a large multicenter, randomized, double-blind study was conducted and found no evidence that ascorbic acid was beneficial for CMT1A patients [166].

Neurotrophins belong to a family of growth factors that support survival and differentiation of neuronal populations and modulate plasticity of the nervous system but also play an important role in the myelination of the peripheral nervous system [167, 168]. A member of this family, ‘Neurotrophin 3’ (NT3), which is known to promote axonal growth and to inhibit myelination during development, has been tested in two different animal models for CMT1A and in one pilot clinical trial conducted on CMT1A patients [169]. NT3 administration resulted in stimulation of axonal regeneration in both animal models. The pilot clinical trial was conducted as a double-blind randomized, placebo-controlled study in which eight CMT1A patients intradermally received either placebo or

NT3 during 6 months. This treatment increased the number of small diameter solitary myelinated fibers in sural nerve biopsies of these patients (considered an indication of axonal regeneration) and reduced sensory loss [169]. However, further studies are required to assess the mode of action of NT3 on myelination and on the peripheral neuropathy in the TrJ mice as administration of exogenous NT3 decreased MPZ levels in sciatic nerves of these mice while suppression of endogenous NT3 signaling enhanced myelin formation [170]. Tyrosine kinase receptor-C (TrkC) is the major target of NT3 while NT3 also binds to another member of the same family, tyrosine kinase receptor-B (TrkB). The altered neurofilament density and phosphorylation found in TrJ mice suggests the involvement of serine/threonine kinase signal transduction. Therefore, agonistic antibodies targeting TrkB and TrkC were tested as possible therapeutic strategy [25, 169]. Treatment of TrJ mice with these antibodies improved CMAP amplitudes, grip strength, regeneration response to injury and the myelination defects [169].

There is currently no evidence for effective drug therapies for axonal forms of CMT. However, our recent study provides some insights into a possible therapeutic strategy for CMT2 and distal HMN. Mutant HSPB1 overexpressing mice developed key features of human CMT2 and distal HMN dependent on the mutation expressed [64]. Overexpression of mutant HSPB1 caused mitochondrial transport defects in cultured DRG neurons which was associated with a decrease of acetylated tubulin levels in peripheral nerve from symptomatic mutant HSPB1-expressing mice. Histone deacetylase 6 (HDAC6) is known for its tubulin deacetylating activity and its role in autophagy [171]. HDAC6 inhibition using small drug-like molecules in mutant HSPB1 mice restored acetylated tubulin levels and the axonal transport defects and induced a significant improvement of the CMT2 phenotype [64]. These data suggest that HDAC6 inhibitors could be used as a possible therapeutic approach for axonal CMT.

HSAN are very rare disorders limiting the development of a specific drug therapy for these patients. Moreover, the incidence of FD is decreasing since genetic prenatal diagnosis is available. Carrier testing helps to avoid or to early terminate affected pregnancies [148]. On the contrary, in case of HSAN type I and FD identification of the specific gene defect and the underlying pathogenic

mechanism facilitates the development of a specific drug therapy, which will be discussed below.

HSAN type I is associated with dominant mutations in SPTLC1 or SPTLC2 [117, 118]. These mutations induce a shift in the substrate specificity of SPTLC1 from L-serine to L-alanine leading to the accumulation of toxic deoxysphingolipids [123]. It was hypothesized that supplementation of L-serine has beneficial effects in the transgenic mouse model for HSAN type I and patients suffering from HSAN type I. Transgenic mice expressing mutant (p.C133W) SPTLC1 received an L-serine enriched diet which reduced levels of the deoxysphingolipid metabolites to wild-type levels. Moreover, motor and sensory performance improved in treated animals. Conversely, administration of an L-Alanine enriched diet worsened the phenotype of p.C133W SPTLC1-expressing mice [172]. Based on these findings, a pilot clinical trial with HSAN type I patients was conducted. Fourteen patients all carrying the same p.C133W mutation in SPTLC1 received either a low or high dose of L-serine during 10 weeks. In both groups, plasma levels showed decreased levels of deoxysphingolipids. Neurological parameters in these patients were not examined. However, some patients reported improvements in relation to sensation, skin, hair and nail problems [172].

In most cases, familial dysautonomia is caused by a homozygous mutation in the IKBKAP gene, leading to partial skipping of exon 20 and a tissue-specific deficiency of IKAP/ELP1. Recently, it has been shown that kinetin (6-furfurylaminopurine), a dietary supplement, is able to rescue the splicing defect and to increase the expression of IKBKAP protein in FD fibroblasts and lymphoblast transformed cell lines. Moreover, animal studies showed that kinetin is well absorbed orally and is distributed in blood plasma and in the CNS and that it is not a clastogenic agent [173]. Following these promising results, an initial clinical trial was conducted in patients homozygous for the most common mutation (IV20+6T>C). This trial showed that the drug was well tolerated and could alter mRNA splicing in FD patients [173].

13.4. ACQUIRED PERIPHERAL NEUROPATHIES

While inherited peripheral neuropathies are the most common inherited disorder of the peripheral nervous system, they only represent a small proportion of

neuropathic patients. Acquired peripheral neuropathies are by far more common. Over 100 different types of acquired neuropathies have been described, and many of them are secondary to other insults (Table **2**).

In this part of the chapter, we will briefly summarize these types and highlight some that might share mechanistic commonalities with inherited peripheral neuropathies (Table **2**).

Table 2: Overview of the different types of acquired peripheral neuropathies

Neuropathy associated with	**Type**
Immunity	Guillain-Barré Syndrome Acquired Chronic Demyelinating Polyneuropathy Sensory and Autonomic Neuropathies Vasculitic Neuropathies Neuropathies associated with Autoimmune Connective Tissue Diseases Sarcoidosis Idiopathic Perineuritis Hypereosinophiilia Syndrome Isaac's Syndrome
Infections	Human Immunodeficiency Virus Leprosy Lyme disease Diphtheria Human T-lymphotropic Virus-1 Cytomegalovirus Epstein-Barr virus Herpes Varicella Zoster virus Hepatitis B and C
Endocrinopathies	Diabetes mellitus Hypoglycemia Acromegaly Hypothyroidism
Systemic disease	Critical illness polyneuropathy Uremic neuropathy Gastrointestinal diseases Liver diseases Chronic Obstructive Pulmonary Disease Gout

Table 2: contd....

Malignancies	Paraneoplastic neuropathies Croptygenic sensory or sensorimotor neuropathy Neuropathy related to tumor infiltration Noninfiltrative neuropathies Acquired amyloidosis Monoclonal gammopathy Bone Marrow transplantation Graft-vs-Host disease
Toxins	Chemotherapy Other medications Industrial and environmental agents Heavy metal intoxication
Nutritional deficiencies	Alcoholic neuropathy Vitamin B (thiamine, pyridoxine, cobalamin) Folic acid Vitamin E Postgastrectomy syndromes Hypophosphatemia Jamaican neuropathy

13.4.1. Neuropathies Associated with Immunity

Immune-mediated polyneuropathies comprise a large group of peripheral neuropathies including Guillain-Barré syndrome and related disorders including 'Acute Motor-Sensory Axonal Neuropathy' (AMSAN) and 'Acute Motor Axonal Neuropathy' (AMAN). Other neuropathies associated with immunological pathogenesis include 'Chronic Inflammatory Demyelinating Polyneuropathy' (CIDP), multifocal motor neuropathy, vasculitic neuropathies, neuropathies associated with autoimmune connective tissue disease (such as rheumatoid arthritis and systemic lupus erythematosus) and sarcoidosis (Table **2**). These disorders are induced by an immune attack against epitopes located in Schwann cells or in the axoplasm depending on the nature (demyelinating or axonal) of the neuropathy. Activated macrophage infiltration and complement activation is frequently observed [174-176].

'Guillain-Barré Syndrome' (GBS) is often referred to by its synonym acute inflammatory demyelinating polyradiculopathy, which is a more descriptive name for the pathogenic process underlying the phenotype. GBS is the most common cause of acute generalized weakness affecting 1-4 per 100,000 annually [177]. GBS can occur at any age, with a peak age of onset in the third to fourth decade of life [177]. In general, affected humans note numbness and tingling in distal parts of both the lower and upper limbs. Progressive muscle weakness is mild but can be severe in such an extent that assisted ventilation is required in 30% of the cases [177]. GBS usually progresses over a period of 2-4 weeks [177]. Most patients gradually recover over several months. Some cases (approximately 5%) die as a result of respiratory distress, pulmonary embolism or cardiac arrhythmias [177].

Two animal models have been developed that mimic some of the key features of GBS. Sensitization of rabbits with ganglioside GM1 or GM1-like lipo-oligosaccharides of a bacterial *Campylobacter* strain from GBS patients caused binding of anti-GM1 antibodies to the nodes of Ranvier and activation of the complement system [178, 179]. These events resulted in the formation of a membrane-attack-complex followed by the disappearance of the sodium channel cluster at the nodes of Ranvier which is in line with the reduced nerve conduction [178, 179]. Secondary axonal degeneration was observed in later stages of the phenotype in this model. The second animal model was generated by the passive transfer of anti-GM1 or anti-GD1 antibodies to mice leading to an axonal GBS-like phenotype [180, 181]. Treatment of these mice with a monoclonal antibody that blocks the cleavage of one of the complement elements prevented dysfunction and structural nerve damage [182].

While the exact nature of the epitope responsible for the immune attack is still unknown, the observations made in the animal models provide evidence in favor of a pathogenic role for anti-ganglioside antibodies and the involvement of the complement system in the underlying mechanism causing GBS. To date, GBS cases are treated by immunotherapy either by plasma exchange (which removes antibodies and the complement system in a non-specific fashion) or by intravenous immune globulin injection (which neutralizes pathogenic antibodies and inhibits autoantibody-mediated complement activation) [177]. A combination

of both plasma exchange and immune globulin treatment was not significantly better than a plasma exchange or immune globulin treatment alone [177].

13.4.2. Neuropathies Associated with Infections

An estimated 50-60% of individuals affected with the human immunodeficiency virus (HIV) developed HIV-associated neuropathy [183]. Of these, many are symptomatic with numbness, pain and paresthesia. The clinical presentation of HIV-associated neuropathy is similar to other forms of acquired neuropathies. It is characterized by a symmetrical and length-dependent degeneration of myelinated and unmyelinated nerve fibers [183]. The neuropathy mainly affects distal sensory nerves, but as disease progresses weakness of the intrinsic foot muscles may occur [183].

The virus, the immune response to the virus and the anti-retroviral drugs (in particular the nucleoside reverse transcriptase inhibitors) are all potentially neurotoxic [183]. These factors could act either alone or in combination to cause the HIV-induced neuropathy [183].

The neurotoxic effect of HIV is probably indirect, as neurons do not express CD4 receptors which are required for viral entry [184, 185]. However, research has focused on HIV gene products, such as the glycoprotein gp120, and their negative effects on various cell types within the nervous system. Local and acute exposure to gp120 induced axonal swelling and increased expression of pro-inflammatory cytokines at the site of application [186].

In vitro studies using primary neurons showed that the neurotoxic effects of gp120 may be directly mediated by the activation of chemokine receptors on the membrane of neurons, or indirectly by activating Schwann cells and peripheral macrophages. Exposure of co-cultures of Schwann cells and DRG neurons to gp120 caused neurite degeneration and neuronal apoptosis [187]. Several reports indicated that the inflammatory mediators released by HIV-infected macrophages may contribute to the indirect neurotoxicity of the virus [185, 188, 189].

Both non-human primate and feline models of HIV (infected with the simian or feline immunodeficiency virus, respectively) showed reduced axonal density and

dying-back of the distal parts of peripheral nerves and accumulating macrophages, confirming the abrogating role of the inflammatory reaction in HIV-associated neuropathy [190, 191].

In addition, there is a growing body of evidence indicating that antiretroviral drugs widely used in the treatment of HIV infections may cause neuropathy, and suggests a pivotal role for mitochondrial dysfunction. Several models, both *in vitro* and *in vivo*, showed that antiretroviral drugs had negative effects on neurite outgrowth, mitochondrial DNA synthesis and neuronal survival [1, 190-195].

Models of virus and drug-induced HIV-associated neuropathy thus provided significant insights into the pathogenic processes underlying the disease. These models support the idea that both direct and indirect mechanisms of viral toxicity are involved, with indirect damage due to inappropriate activation of inflammatory cells and subsequent release of inflammatory cytokines in peripheral nerves. To date, potential targets to either prevent or attenuate the development of the neuropathy or to treat symptoms of the neuropathy have not yet been identified. However, the animal models that are currently used in this field of research could provide excellent tools to investigate and identify new and efficacious targets to treat this debilitating neuropathy.

13.4.3. Neuropathies Associated with Endocrinopathies

Diabetic neuropathy is a common and severe complication that is more persistent in type 2 than in type 1 diabetes [196]. Approximately 50-60% of diabetic cases develop progressive length-dependent peripheral axonal loss in a distal to proximal pattern [196-198]. Large myelinated and unmyelinated sensory axons are predominantly affected [196-198]. Symptomatic muscle weakness tends to develop in later disease stages [199]. Reduced blood flow due to loss of autonomic nerve fibers may contribute to the development of diabetic neuropathy [196]. Diabetic neuropathy is the leading cause of diabetes-related hospital admissions [197]. Both hyperglycemia and dyslipidemia are associated with diabetes [197]. Both are thought to cause aberrant alterations in energy balance and metabolism leading to cellular injury and neuropathy [196, 197]. However, there are no experimental data that confirm changes in bioenergetics as the cause of diabetic neuropathy [196, 197].

In diabetes, the high oxidative environment and the accumulation of carbohydrates accelerate the formation of 'Advanced Glycation End-products' (AGEs) [200]. Advanced glycation is a non-enzymatic process that chemically modifies proteins, lipids and nucleic acids by adding reactive carbohydrate groups to exposed sites [196]. AGEs can cause the development of diabetic neuropathy in two ways. First, advanced glycation tends to decrease the biological function of proteins, thus inhibiting neuronal activity [201]. Second, extracellular lipid and protein AGEs bind to cell surface receptors, particularly the receptor for AGE (RAGE), initiating inflammatory signaling cascade that further increases oxidative and nitrosative stress and increases neuronal cell injury [202, 203]. The importance of AGE in the development of diabetic neuropathy has been confirmed using AGE inhibitors and through studies of RAGE knockout mice [204, 205]. Therapeutically targeting RAGE or targeted disruption of RAGE significantly reduced diabetic neuropathy in mice [206]. Although the exact functional role of RAGE is unknown, these data could indicate that AGEs are a valid therapeutic target to prevent or reverse diabetic neuropathy.

While neurons are not relying on insulin signaling for glucose uptake, there is evidence suggesting that peripheral insulin resistance might contribute to neuropathy [207]. Application of insulin to primary cortical neurons blunted the cellular signaling in response to subsequent exposure to insulin [208]. Furthermore, clinical data indicate a positive correlation between the degree of insulin resistance and the onset of complications (including neuropathy) independent of glycemia levels.

Current treatment strategies consist of targeting hyperglycemia and pain management [196]. Several other therapeutic interventions are being investigated including cytoprotective therapies (reducing cell death, providing trophic support), therapies that inhibit the NADPH oxidase (a key enzyme in the generation of oxidative and nitrosative stress in diabetic neuropathy) and therapies that reduce neuro-inflammation [196].

13.4.4. Neuropathies Associated with Systemic Disease

In this part, we will discuss neuropathy associated with critical illness as an example of systemic disease-associated neuropathies (Table **2**). 'Intensive Care

Unit-Acquired Weakness' (ICUAW) is an increasingly recognized and important clinical consequence of critical illness characterized by skeletal muscle wasting and weakness [209, 210]. In the most severe cases, complete paralysis occurs and 30-40% of patients in the intensive care unit develop long-term disability [209, 210]. A significant number of patients who are intubated for more than 7 days (52-57%), and many patients with sepsis or systemic inflammatory response syndrome (68-100%) develop ICUAW [209-211].

It has been suggested that many factors contribute to ICUAW including neuropathy, altered muscle integrity, sarcoplasmic reticulum dysfunction, electrical in-excitability and finally bioenergetic failure and oxidative stress [209]. Based on electrophysiological findings, ICUAW patients predominantly display axonal loss rather than prolonged conduction times [209]. However, given the rapid course of symptom onset after sepsis, it is unlikely that the loss of muscle mass is only due to neuropathy [209]. Moreover, despite the reduction in amplitudes of the CMAPs and SNAPs, nerve histology is in general normal suggesting that there are functional rather than structural abnormalities [212, 213]. As a consequence, it is generally accepted that ICUAW results from both neuronal and muscular damage [209].

13.4.5. Neuropathies Associated with Nutritional Deficiencies

As a typical example of a neuropathy associated with "nutritional deficiencies", we will focus on alcoholic neuropathy (Table **2**). Peripheral neuropathy is a frequent complication (12-50%) of chronic alcohol abuse [214]. Alcoholic neuropathy is characterized by a symmetric polyneuropathy pattern predominantly affecting the lower extremities [214]. Clinically, positive sensory signs (including hyperalgesia) and weakness in distal parts of the lower extremities are common features of alcoholic neuropathy [214]. Distal axonal loss involves both myelinated and unmyelinated fibers [214]. Although the neuropathy develops slowly, a combination of malnutrition and alcoholism may accelerate the progression [214].

Both *in vitro* and *in vivo* studies showed that long-term exposure to ethanol reduces axonal transport [215, 216]. Exposure to ethanol caused a reduction in neurofilament abundance and an increase in phosphorylated neurofilaments [217,

218]. Phosphorylation of microtubule-associated proteins was also altered by ethanol [219] and hepatoma-derived cells showed changes in acetylation of microtubules after exposure to alcohol [220].

Ethanol also modulates intracellular signaling cascades involving 'Protein Kinase A' (PKA) and 'Protein Kinase C' (PKC) [221]. As both enzymes have been implicated in pain responses, they may be associated with painful symptoms in alcoholic neuropathy [222]. Inhibition of PKC attenuated hyperalgesia in a rat model of alcoholic neuropathy [223].

Vitamin B has also been tested for treating peripheral neuropathy [224]. However, there is insufficient evidence to determine whether vitamin B is beneficial.

13.4.6. Neuropathies Associated with Chemotherapy

Induction of peripheral neuropathy is a common complication of chemotherapy to treat several types of malignancies (Table **2**). Despite the high prevalence, little is known about the underlying mechanism causing chemotherapy-induced neuropathies. Chemotherapy usually causes sensory neuropathy, and involvement of motor or autonomic modalities depend on the compound used for chemotherapy [225-227]. Commonly, induction of neuropathy is apparent weeks to months after (first) exposure and may continue despite drug withdrawal [225-227]. In general, the neurotoxic effects of chemotherapy are reversible when recognized early during treatment [225-227]. Progressive weakness, paresthesia and loss of deep tendon reflexes are common clinical features. Sensory impairment and autonomic deficits including impotence and orthostatic hypotension may occur. Severe irreversible damage can occur if drug treatment is continued [225-227]. Three typical examples of chemotherapy-induced neuropathies include vincristine-, taxane- and bortezomib-induced neuropathies. Overall, these drugs induce an axonal neuropathy in a dose-dependent, cumulative manner [225-227]. In most cases, axonal degeneration is mild and complete regeneration may occur after drug withdrawal. Demyelinating neuropathies resulting from drug toxicity are far less common [225-227].

Vincristine is a well-recognized neurotoxic chemotherapeutic agent. This compound specifically binds to tubulin and blocks its polymerization into

microtubules, arresting in this way mitosis in metaphase. Taxanes (paclitaxel (Taxol®) and docetaxel (Taxotere®)) are anti-neoplastic compounds that promote the assembly of microtubules and stabilize their formation by preventing de-polymerization. Intravenous injection of vincristine in rabbits caused accumulation of organelles at the nodes of Ranvier in sciatic nerves [228, 229]. Subsequent degeneration of organelles is thought to contribute to axonal degeneration after vincristine treatment [230]. Other studies using rats showed that intra-neural administration of vincristine caused a reversible blockade of retrograde axonal transport mediated by dynein (a gene associated with IPNs) [231]. Similarly, sub-epineural injection of Taxol completely inhibited slow axonal transport of tubulin in rat sciatic nerves, while transport of neurofilaments was unaffected [232]. These observations were the first to suggest that slow transport involves an equilibrium between polymerized and depolymerized forms of axonal cytoskeletal structures. Fast axonal transport (such as transport of lysosomes, vesicles and mitochondria) seems to be largely unaffected by taxol [232]. It was also suggested that taxol affects fast axonal transport in early stages (immediately following administration) due to structural blockade of accumulating tubulin polymers [233, 234].

Bortezomib is a proteasome inhibitor that has a beneficial effect against recurrent or newly diagnosed multiple myeloma [235]. Bortezomib is responsible for a predominant small fiber painful axonal sensory distal neuropathy [235]. As an inhibitor of the proteasome, bortezomib causes the accumulation of toxic (aggregated) proteins eventually leading to cell death. Furthermore, up-regulation of pro-apoptotic proteins, down-regulation of several proteins involved in DNA repair pathways and induction of the unfolded protein response have been observed in pre-clinical studies [236]. Furthermore, proteasome inhibition impaired microtubule nucleation and organization suggesting that axonal transport defects and cytoskeletal disorganization might contribute to the development of bortezomib-induced neuropathy [237]. Interestingly, several genes encoding proteins involved in proteolytic pathways have been associated with motor neuron disorders and IPNs (such as ‘Ubiquilin 2’ in amyotrophic lateral sclerosis and the E3 ubiquitin ligase LRSAM1 in axonal CMT). Finally, inhibition of the proteasome system activates HDAC6 which plays a crucial role in axonal

transport and in autophagy [171]. All together, these observations suggest that proteasomal inhibition might affect axonal integrity in chemotherapy-induced neuropathies.

CONCLUSION AND FUTURE PERSPECTIVES

So far, numerous genes underlying IPNs have been identified and the list of candidate causative genes and loci is still increasing. A lot of research is ongoing to find the exact pathogenic mechanisms of these mutant genes and how this leads to different subtypes of IPNs. This is challenging since IPNs are characterized by a large clinical and genetic heterogeneity. Similarly, a very wide range of APNs have been described, and most of these show clinical heterogeneity. Moreover, the exact pathogenic mechanism underlying these neuropathies is still poorly understood for most of them. We are convinced that the development of cell culture and animal models for both inherited and acquired peripheral neuropathies is necessary and will help in the search for common underlying pathogenic mechanisms. Hopefully, the identification of these common pathways will pave the way for the development of therapeutic strategies.

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CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

LIST OF ABBREVIATIONS

AARS = Alanine-tRNA synthetase

AGE = Advanced Glycation End-products

AMAN = Acute Motor Axonal Neuropathy

AMSAN = Acute Motor-Sensory Axonal Neuropathy

APN = acquired peripheral neuropathies

ARS = aminoacyl-tRNA synthetase

ATL1 = Atlastin-1

ATSV = Axonal Transport of Synaptic Vesicles

CIDP = Chronic Inflammatory Demyelinating Polyneuropathy

CMAP = compound muscle action potential

CMT = Charcot-Marie-Tooth disease

CNS = central nervous system

Cx32 = Connexin-32

DARS = Aspartyl-tRNA Synthetase

DCTN1 = Dynactin subunit 1

DI-CMT = Dominant Intermediate Charcot-Marie-Tooth disease

Distal HMN = distal Hereditary Motor Neuropathy

DNMT1 = DNA-methyltransferase 1

DRG = Dorsal Root Ganglia

DRP2 = Dystrophin-Related Protein 2

DYNC1H1 = Dynein, Cytoplasmic 1, Heavy chain 1

EGR2 = Early Growth Response 2

ELP1 = Elongator Complex Protein 1

ENU = N-Ethyl-N-nitroso-ureum

FD = Familial Dysautonomia

FH2 = Formin Homology 2

GARS = Glycyl-tRNA synthetase

GBJ1 = Gap-Junction Bèta-1

GBS = Guillain-Barré Syndrome

GDAP1 = Ganglioside-induced Differentiation-associated Protein-1

HDAC6 = Histone deacetylase 6

HIV = Human Immunodeficiency Virus

HMSN = Hereditary Motor and Sensory Neuropathies

HSAN = Hereditary Sensory and Autonomic Neuropathy

HSN = Hereditary Sensory Neuropathies

HSP = Hereditary Spastic Paraplegia

HSPB1 = small Heat Shock Protein B1

HSPB3 = small Heat Shock Protein B3

HSPB8 = small Heat Shock Protein B8

ICUAW = Intensive Care Unit-Acquired Weakness

IKBKAP = IκB Kinase Complex-associated Protein

INF2 = Inverted Formin 2

IPN = inherited peripheral neuropathies

KARS = Lysine-tRNA synthetase

KIF1A = Kinesin-like protein 1A

LITAF = Lipopolysaccharide-Induced Tumor Necrosis Factor-Alpha Factor

LMNA = Lamin A/C

MAL = myelin and lymphocyte protein

MFN2 = Mitofusin-2

MPZ = Myelin Protein Zero

MTMR13 = Myotubularin-related Protein 13

MTMR2 = Myotubularin-related Protein 2

NCV = nerve conduction velocities

NDRG1 = N-myc Down-Regulated Gene-1

NEDD4 = Neural Precursor Cell Expressed, Developmentally Down-regulated 4

NEFL = Neurofilament Light-chain

NT3 = Neurotrophin 3

NTRK1 = Neurotrophic Tyrosine Kinase Receptor, type 1

PI-3,5-P2 = phosphatidyl-inositol 3,5-bisphosphate

PI-3-P = phosphatidyl-inositol 3-phosphate

PKC = Protein Kinase C

PMP22 = Peripheral Myelin Protein 22

PNS = peripheral nervous system

PRX = Periaxin

RAB7 = Ras-related Protein Rab-7

RAGE = Receptor for AGE

RI-CMT = Recessive Intermediate Charcot-Marie-Tooth disease

SH3TC2 = SH3 Domain and Tetratricopeptide Repeat-containing Protein 2

SIMPLE = Small Integral Membrane Protein of Lysosome/late Endosome

SNAP = sensory nerve action potential

SPT = Serine Palmitoyltransferase

SPTLC1 = Serine Palmitoyltransferase Long Chain subunit 1

SPTLC2 = Serine Palmitoyltransferase Long Chain subunit 2

SPTLC3 = Serine Palmitoyltransferase Long Chain subunit 3

Tr = Trembler

Tr-J = Trembler J

TrkA = Tyrosine kinase receptor-A

TrkB = Tyrosine kinase receptor-B

TrkC = Tyrosine kinase receptor-C

TSG101 = Tumor Susceptibility Gene 101

UK = United Kingdom

US = United States

WNK1 = Protein kinase with no lysine 1

YARS = Tyrosine-tRNA synthetase

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Section 4: Future Therapies

CHAPTER 14

Targeting Molecular Mechanisms of Cognitive Dysfunction

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Abstract: Increasing knowledge of the neurobiological basis of synaptic plasticity and memory has opened new venues for the development of cognitive-enhancing drugs that could be used in the treatment of memory loss associated with neurological and psychiatric disorders. Neuromodulatory systems influencing memory formation include stress hormones as well as a range of neurotransmitter and neuropeptide signaling pathways. Here, we review some of the findings on memory enhancement by drugs acting on neuromodulatory systems and discuss the possible implications for the development of cognitive enhancers.

Keywords: Synaptic plasticity, Glutamate, Noradrenaline, Dopamine, Acetylcholine, Catecholamine, Protein kinase, Neuropeptide, Neurotrophin, Neurotransmitter, Neuronal receptor, Transcription factor, Epigenetics, Neuromodulation, Learning, Memory modulation, Memory consolidation, Cognitive dysfunction, Cognitive enhancement, Translational research.

14.1. INTRODUCTION

Deficits in cognitive function accompany many neurodegenerative, neurodevelopmental, and psychiatric disorders, including Alzheimer's (AD),

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Parkinson's (PD), and Huntington's diseases (HD), autism, and schizophrenia. In addition, moderate memory impairment during aging (mild cognitive impairment, MCI) might represent an intermediate state between normal aging and dementia and indicate the onset of AD. Memory loss associated with aging and neurological disorders constitutes a growing unmet medical need for which no adequate treatment is yet available. Cognitive enhancement through pharmacological stimulation of neuronal mechanisms involved in synaptic plasticity and memory formation has been put forward as a strategy to improve memory dysfunction. The advancement in our understanding of the molecular basis of neural plasticity and memory has opened many avenues for the discovery and development of cognitive-enhancing drugs. In this chapter, we review some of the therapeutic targets currently under investigation for the development of candidate cognitive enhancers, focusing on neuromodulatory mechanisms that regulate memory formation.

Most of the evidence reviewed here was provided by preclinical research using animal models, in which behavioral outcomes assumed to represent specific aspects of memory are measured. Although the translation from preclinical experiments to the clinical setting in this field is rather complex and has important limitations, these models have allowed the characterization of mechanisms and pathways, both at the molecular and brain systems levels, that can be used as targets for cognitive enhancement.

14.2. MOLECULAR BASIS OF SYNAPTIC PLASTICITY AND MEMORY

The current view of memory formation and storage as a process resulting from modifications in the strength of synaptic connections has originally emerged from the *cellular connectionist approach* proposed by Santiago Ramon y Cajal, and was further developed as a model of learning based on synaptic plasticity by Konorski and Donald Hebb in the late 1940s (reviewed in [1]). A seminal experimental demonstration of synaptic plasticity was provided by Bliss and Lomo in 1973. They showed a persistent increase in synaptic response in the hippocampus as a result of high-frequency stimulation, a phenomenon named long-term potentiation (LTP) [2]. Over the past decades, a consistent body of evidence has indicated that synaptic plasticity processes based on, or similar to,

LTP mediate the formation and storage of many types of memory. Moreover, the use of a variety of model organisms including the fruit fly *Drosophila*, the sea snail *Aplysia*, rats, and mice has significantly contributed to identify highly conserved basic molecular mechanisms underlying or influencing neural plasticity and memory [1, 3, 4]. The introduction of transgenic and knockout mouse models, including spatially and temporally restricted modifications, contributed to the identification of molecular pathways mediating LTP and memory, allowing the examination of the consequences of genetic disruption or stimulation of selective mechanisms in discrete neuronal populations [5, 6].

The hippocampus, a brain area first shown to be involved in the formation of new memories by human studies conducted in the 1950s by Brenda Milner (reviewed in [7], has been the target of many preclinical studies on synaptic plasticity and memory. The induction of LTP in the rat dorsal hippocampus was prevented by blocking the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor, which is associated with a channel permeable to calcium and sodium ions. Importantly, intracerebral administration of an NMDA receptor antagonist impaired hippocampus-dependent spatial memory in rats, indicating that hippocampal NMDA receptor activation is required for both LTP and memory formation [8]. Extensive evidence now indicates that activation of NMDA receptors, along with other types of glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, AMPA, and metabotropic, (mGluR, receptors), constitute a trigger that can initiate a sequence of downstream signaling events underlying synaptic plasticity and learning [9].

Downstream of NMDA receptor activation, protein kinase cascades are stimulated, leading to transcription factor phosphorylation, expression of immediate-early genes, and *de novo* protein synthesis. Protein kinase pathways with established roles in LTP and memory include the calcium-calmodulin-dependent protein kinase II (CaMKII), phospholipase C (PLC)/protein kinase C (PKC), cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP response element binding protein (CREB), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK), and phosphatidylinositol 3-kinase (PI3K) pathways. Immediate-early genes associated with memory formation include c-fos, Arc, and zif268. Other mechanisms that

can contribute to this process include brain-derived neurotrophic factor (BDNF), acting on TrkB, which is a member of the receptor tyrosine kinase (RTK) family. The ultimate outcome of this cascade of biochemical events and alterations in gene expression is the reinforcement of selective subgroups of synapses, and the growth of new synaptic connections [3, 4, 6, 10-12]. The initial phase of memory formation after learning, classically considered to last for up to a few hours, is called *consolidation*. The long-term persistence of memory after the initial consolidation stage involves additional mechanisms, including the atypical protein kinase C isoform, protein kinase Mzeta (PKMz) [13].

On the basis of the knowledge on crucial molecular components of memory formation, pharmacological cognitive-enhancing strategies have been developed and tested. For example, drugs that stimulate NMDA and AMPA glutamate receptors have been investigated as memory facilitators in both animal and human studies. The drug d-cycloserine, which stimulates NMDA receptors by acting as a partial agonist at its glycine binding site, has been evaluated in Alzheimer's disease patients [14, 15]. Ampakines, which stimulate AMPA receptors, have also been developed as candidate cognitive enhancers [16, 17]. However, there are several challenges for the development of clinically acceptable glutamate receptor stimulators, including the risk of facilitating neuronal damage by excessive NMDA receptor stimulation, through the process known as excitotoxicity [18]. Memantine, a noncompetitive NMDA receptor antagonist currently used in the treatment of Alzheimer's disease, probably have neuroprotective and antioxidant actions that result in beneficial effects on cognition [19, 20].

14.3. NEUROMODULATORY MECHANISMS REGULATING MEMORY FORMATION

The molecular mechanisms and events described above are believed to be part of the *core* set of neural plasticity events underlying memory formation. However, memory strength is also influenced by a variety of other signaling molecules and pathways. Evidence that memory consolidation in animals can be enhanced by administration, after learning, of a number of chemical agents has consistently indicated that the mammalian brain has endogenous systems that enable the enhancement of emotionally significant memories. For example, hormones

released in response to novel and arousing experiences, including epinephrine and corticosterone, vasopressin, and beta-endorphin, directly or indirectly regulate brain activity after learning to stimulate the mechanisms related to memory consolidation. Also, a number of neurotransmitters (including catecholamines and acetycholine) and neuropeptides, as well as epigenetic mechanisms regulating gene expression at the nuclear level, such as chromatin-modifying enzymes, can be considered regulatory mechanisms that influence neuronal gene expression and alter synaptic plasticity, resulting in enhanced memory strength [4, 21, 22]. Below we will focus on selected examples of cognitive enhancement based on stimulation of some of these modulatory systems. Many other neurotransmitter and signaling pathways play a modulatory role in memory formation and have been investigated as targets for cognitive enhancement. Current targets include receptors and transporters for GABA, cannabinoids, serotonin, glucocorticoids, and adenosine. Fig. **1** shows a schematic of selected mechanisms influencing memory formation.

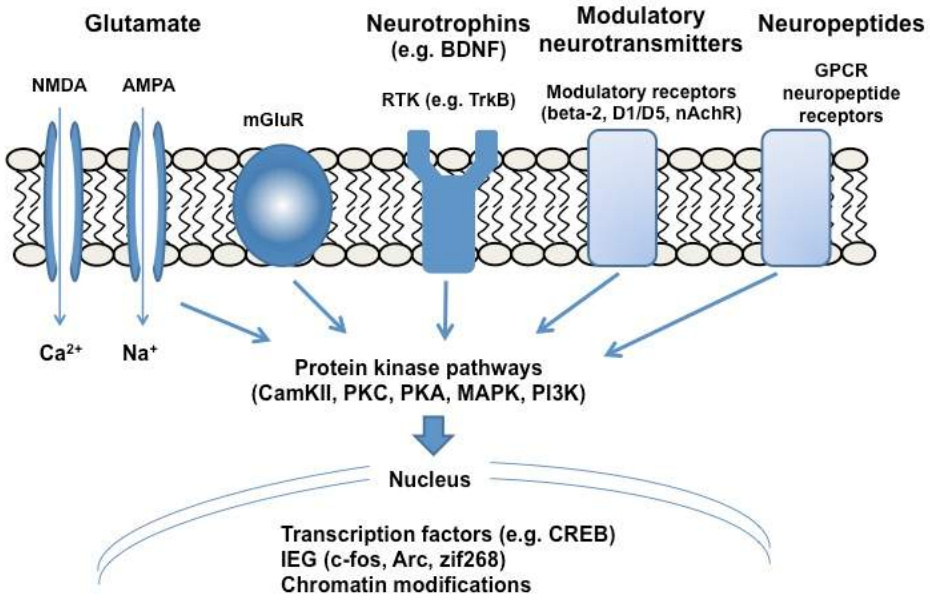

Figure 1: Selected molecular mechanisms that are involved in underlying and regulating memory formation and can be targeted for the development of cognitive enhancers.

Glutamate activates NMDA along with AMPA and mGluR receptors, triggering cellular calcium entry and stimulation of protein kinase pathways including CaMKII, PLC/PKC, cAMP/PKA/CREB, MAPK, and PI3K. This leads to activation of transcription factors incuding CREB and IEGs such as c-fos, Arc, and zif268. At the nuclear level, gene expression related to memory formation is facilitated by chromatin relaxation induced by histone acetylation. This cascade of molecular events is regulated by a range of mechanisms including neurotrophins acting on the Trk members of the RTK family, noradrenaline activation of beta-2 receptors, dopamine acting on D1/D5 receptors, acetylcholine stimulation of nAchRs, and GPCRs activated by neuropeptides. Targeting these systems in experimental models in a way that stimulates excitatory receptors, protein kinase signaling, and gene expression, leads to memory enhancement and amelioration of cognitive deficits associated with brain disorders.

14.4. COGNITIVE ENHANCERS TARGETING MEMORY MODULATORY PATHWAYS

14.4.1. Catecholamine Receptor Systems and the cAMP/PKA/CREB Pathway

It has been known since the 1970s that epinephrine injections after learning can facilitate memory in rats [23]. The effects of epinephrine on memory are probably mediated by the ascending vagus nerve and result in activation of the noradrenergic system in the brain. Stimulation of the noradrenergic system within the basolateral amygdala (BLA) is required for peripheral epinephrine to enhance memory [24]. Intracerebral administration of noradrenaline in selected brain areas including the hippocampus and the BLA can also enhance memory consolidation [25]. Moreover, rats given epinephrine injections show a more persistent memory retention compared to control animals [26].

This evidence strongly indicates that central activation of the noradrenergic system, either directly or triggered by peripheral epinephrine release, can enhance memory formation and persistence. Thus, drugs stimulating noradrenergic receptors might be investigated as potential cognitive enhancers. In fact, memory in rats can be enhanced by administration, into the BLA, of the beta-2 adrenoceptor agonist clenbuterol, the alpha-2 adrenoceptor antagonist idazoxan,

or the non-selective alpha-adrenoceptor agonist phenylephrine combined with the alpha-2 adrenoceptor antagonist yohimbine [27-29].

In addition to noradrenaline, another catecholamine, dopamine, has been shown to importantly influence memory formation. Early studies showed memory-enhancing effects of amphetamine, which acts partially by stimulating dopaminergic transmission, and the effects of agents that act at specific subtypes of dopamine receptors have been investigated. Memory consolidation in rats is increased by intracerebral administration of the D1/D5 receptor agonist SKF 38393 [30, 31]. SKF 38393 also rescues age-related deficits in synaptic plasticity and memory in mice [32]. In contrast, when pharmacological manipulation of D2 and D3 receptor antagonists is used, often memory enhancement is observed after receptor blockade by antagonists [33, 34]. About ten years ago, some of the leading candidate cognitive-enhancing drugs targeting dopamine receptors included dihydrexidine, a D1/D5 receptor agonist, and A-412997, a D4 receptor agonist [35-38]. Currently many other agonists and antagonists at D1/D5 receptors are available and under investigation (see [39-41] for further details).

Noradrenaline and dopamine receptors are coupled to stimulation of the cAMP/PKA/CREB signaling pathway downstream of receptor activation. Agents that act intracellularly to directly stimulate this pathway are among the first candidate drugs developed with the aim of ameliorating cognitive function in humans [42-45]. Activation of beta-adrenergic or D1/D5 receptors leads to increases in cAMP levels as a result of enhanced adenylyl cyclase activity. cAMP in turn activates PKA, which recruits MAPK and translocates to the nucleus, phosphorylating and activating the transcription factor CREB, resulting in altered gene expression.

Cognitive-enhancing drugs stimulating the cAMP/PKA/CREB pathway include phosphodiesterase type 4 (PDE4) inhibitors. PDE4 is an enzyme that catalyzes hydrolysis of cAMP. Rolipram, a prototypical PDE4 inhibitor widely used over the last fifteen years in studies focusing on memory and LTP enhancement, ameliorates memory deficits in rodent models of cognitive dysfunction [32, 46-49]. More recently, studies in rats and mice shown similar enhancing effects of newer drugs that target PDE5, such as zaprinast and icariin [50-52].

14.4.2. Cholinergic Receptors

Acetylcholinesterase inhibitors (AChEi) including donepezil, rivastigmine, and galantamine are established in the treatment of Alzheimer's disease. In experimental animals, memory consolidation can be enhanced by AChEis or muscarinic cholinergic receptor agonists (*e.g.*, oxotremorine) [53]. Also, on the basis of the well-documented cognitive-enhancing effects of nicotine, other drugs that stimulate nicotinic cholinergic receptors have been developed as potential memory enhancers [54].

14.4.3. Neuropeptide Systems

Neuropeptides, which can be released from neurons as co-transmitters, represent an important class of neuromodulatory molecules in the brain. Several neuropeptides have been shown to regulate memory formation and expression, including neuropeptide Y, neuropeptide S, vasoactive intestinal peptide (VIP), somatostatin, substance P, vasopressin, adrenocorticotropin (ACTH), cholecystokinin (CCK), oxytocin, galanin, corticotrophin-releasing hormone (CRH), bombesin-like peptides, and endogenous opioids such as beta-endorphins. Most neuropeptides act by activating membrane receptors of the G protein-coupled (GPCR) receptor family, leading to stimulation of intracellular protein kinase signaling pathways [55-59].

A number of recombinant neuropeptides and synthetic peptides are currently available as experimental cognitive enhancers. As an example, recombinant bombesin (an amphibian peptide that displays biological actions similar to its homolog gastrin-releasing peptide (GRP), a mammalian neuropeptide) enhances hippocampal memory retention in rats and rescues a memory deficit produced by beta-amyloid peptide (25-35) in the hippocampus, whereas a GRP receptor antagonist impairs memory [60, 61].

14.4.4. Chromatin-Modifying Mechanisms

Signaling triggered by neuronal receptors and mediated by intracellular pathways ultimately leads to alterations in gene expression. In the nucleus, the levels of gene expression are also regulated *epigenetically* by an additional set of mechanisms. Epigenetic regulation involves chromatin remodeling, histone

modification, and DNA methylation. One of the more exciting recent advances in the field of experimental drug-induced cognitive enhancement was the discovery that agents that act at the epigenetic level to facilitate gene expression, particularly by inducing chromatin relaxation through inhibition of histone deacetylases (HDACs), can enhance memory formation. Histone acetylation increases accessibility for transcriptional regulatory proteins, whereas deacetylation mediated by HDACs promotes chromatin condensing and reduces transcription. Increased histone acetylation might be a molecular feature associated with stronger memories [62], and systemic or intracerebral administration of HDAC inhibitors including sodium butyrate, trichostatin A, and valproic acid enhances memory and rescues experimental memory loss in rats and mice [63-66]. The memory-enhancing effects of HDACis might be mediated primarily by target genes regulated by the CREB:CREB-binding protein (CBP) transcriptional complex [67].

14.5. THE CHALLENGE OF TRANSLATING EXPERIMENTAL MEMORY ENHANCEMENT TO THE CLINICAL SETTING

As illustrated by the examples reviewed above, experimental drug-induced memory enhancement in animals can be relatively easily obtained in the laboratory through the manipulation of a range of neurochemical systems. However, and despite the growing need for therapies that can alleviate the cognitive dysfunction associated with brain disorders, so far this extensive body of neurobiological research has failed to deliver clinically successful cognitive enhancers developed on the basis of the biology and neurochemistry of memory formation. Several factors contribute to the high complexity of translating findings from the bench to patients in this field. First, although the fundamental brain processes underlying neural plasticity and memory are highly conserved across species, cognitive processing and dysfunction in humans is clearly much more complex than in animals, and experimental models focus on very specific aspects of memory function [68]. One obvious challenge is to rescue memory loss in a selective way that is beneficial for the patient, without at the same time promoting the storage or expression of unnecessary and unwanted (*e.g.*, traumatic) information. Second, drugs acting on neuromodulatory systems can produce different, and under some conditions opposite, effects on the different

phases and types of memory. For example, stimulators of noradrenergic or glucocorticoid receptors typically enhance memory formation when given after training but can impair memory expression before retrieval. Finally, the mnemonic effects of neuromodulatory drugs can be affected by relatively subtle aspects related to learning, such as novelty, contextual information, and stress levels. These limitations will need to be addressed to allow the significant advances in the knowledge on the fundamental mechanisms of memory to give rise to new treatments for cognitive dysfunction.

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CONFLICT OF INTEREST

The author(s) confirms that this chapter contents have no conflict of interest.

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CHAPTER 15

Stem Cell Applications for Neurodegenerative Diseases

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Abstract: Notwithstanding the past decades of research, efficient treatments for neurodegenerative diseases do not exist. However, stem cell therapies have become increasingly attractive options for a broad spectrum of human neurodegenerative diseases. Diverse classes of stem cells, such as embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) can be useful as a source material for understanding the basic biology of cellular differentiation, disease modeling, and provide novel sources for autologous cellular therapies in neurodegenerative diseases. Indeed, the transplantation of stem cells or their derivatives and the mobilization of endogenous stem cells have been proposed in animal models of neurodegenerative disease as therapeutic mechanisms to restore function. In this chapter, we discuss some general issues relating to the scientific basis of stem cell–based therapies and their prospects in neurological disorders including Parkinson's disease, Alzheimer's disease, Huntington's disease and Amyotrophic lateral sclerosis.

Keywords: Alzheimer's disease, Amyotrophic lateral sclerosis, embryonic stem cells (ESCs), Huntington's Disease, induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), Neural stem cells (NPCs), Neurotrophic factors, Parkinson's Disease, neural progenitor cells, stem cells, treatment, cell therapy.

15.1. INTRODUCTION

Despite decades of research, effective treatments for neurodegenerative diseases do not exist. However, cellular therapies present themselves as new attractive options and the application of stem cell research for a broad spectrum of human neurodegenerative diseases is rapidly growing.

As the functional units for growth and regeneration in many, though not all tissues, stem cells hold a position of significant importance for maintaining proper

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tissue function. Stem cells are defined as undifferentiated cells with prolonged self-renewal capacity, and depending on their origin, are able to differentiate into multiple cellular lineages or all cell types of the body [1]. Mammalian stem cells can be classified according to cell types that they can generate, and by the strategy used for their derivation. Stem cell classes comprise of embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) [2, 3].

These diverse classes of stem cells can be a useful source material for understanding the basic biology of cellular differentiation, disease modeling, and provide novel sources for autologous cellular therapies in neurodegenerative diseases.

ESCs are derived from the inner cell mass of a developing blastocyst (early embryo) and can be experimentally isolated from mouse, monkey and human. These cells may be expanded in culture while retaining pluripotency and possess the capacity to give rise to various organs and tissues [4, 5]. When moral and ethical reasons are taken into account, the use of human embryonic stem cells in disease therapy is not justified, since other alternatives for regenerative medicine are already available and will be discussed below.

NSCs can be isolated from the fetal, neonatal, and adult neural tissues and propagate in culture [6, 7]. NSCs are multipotent cells capable to differentiate into three major cell types of Central Nervous System (CNS), neurons, astrocytes and oligodendrocytes. In humans, neuronal precursor cells in the adult brain have been found at two major NSC niches: the dentate gyrus of the hippocampus and the subventricular zone of the olfactory bulb, although a very small number of stem cells might also exist in other brain regions [8]. The proliferation and differentiation of NSCs are precisely regulated by a complex system composed of a large number of morphogens, growth factors, surrounding cells, transcription factors, among others. Neurons derived from these cells contribute to learning, memory, and the autonomous repair of the brain under pathological conditions (for review see [1]). Indeed, alterations in adult neurogenesis are frequently observed in neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington´s disease (HD) and the transplantation

of neurons obtained from NSCs cultured *in vitro* have been used for cellular replacement in neurological diseases to ameliorate neurodegeneration [1].

MSCs can easily be obtained from various tissues, such as bone marrow, adipose tissue, placenta and umbilical cord, cartilage, and expanded vigorously until the tissues differentiate into specific cell lineages [9-19]. Transplantation of MSCs into the injured brain may provide therapeutic benefits. MSCs transplanted into the brain have been demonstrated to promote functional recovery by producing trophic factors that induce survival and regeneration of host neurons. MSCs are immunocompatible by nature and there are no ethical issues related to their use [20].

Recently, a new class of pluripotent stem cells, iPSCs (induced pluripotent stem cells), has been generated from adult somatic cells such as fibroblasts by introduction of embryogenesis-related transcription factors [21]. The original strategy utilized Oct 3/4, Klf, Sox2 and c-Myc [21], and various groups are now reprogramming adult somatic cells using several approaches to delivery embryonic transcription factors such as vector, virus, protein or RNA [22-24]. The iPSCs share many properties with human ESCs, such as morphology, the ability of indefinite growth and pluripotency, but they are not identical since they display differences in gene expression signatures [25]. iPSCs now can be derived from a variety of cell lineages and are able to differentiate into certain cell types including neurons. Indeed, patient-specific iPSCs are already applied in disease modeling, drug testing and discovery and provide novel sources for autologous cellular therapies [26]. Hence, cellular reprogramming is the most promising approach due to the fact that it allows the generation of patient-specific stem cells, which in turn can be differentiated in neural lineages and open a vast new territory in looking for effective treatments for neurological diseases.

More recently, a study has shown that either human fibroblasts or iPSCs can efficiently be converted into functional induced neuronal cells (iNCs) through a forced expression of a combination of three neural lineage-specific transcription factors, Ascl1, Brn2 and Myt1l [27]. Moreover, human fibroblast can be effectively reprogrammed into dopaminergic neuron-like cells by expression of five transcriptional factors Mash1, Ngn2, Sox2, Nurr1, and Pitx3 [28]. Concerning possible clinical applications, the major disadvantage of iNCs is the

lack of expandability, generating a limited cell number. Indeed, several open questions have arisen with iNCs, but they represent great promises for both cell replacement and cell modeling of neurodegenerative diseases.

Indeed, the transplantation of stem cells or their derivatives and the mobilization of endogenous stem cells have been proposed in animal models of neurodegenerative disease as therapeutic mechanisms to restore function [29]. For instance, it might be possible to replace lost neurons or glial cells by transplantation of stem cell pre-differentiated *in vitro* to different stages of maturation. The stimulation of endogenous neural stem cells to form new neurons or glial cells in the adult CNS can represent an alternative for cell replacement. Additionally, a functional enhancement might be achieved with the release of neuroprotective molecules by grafted stem cells [30].

There is also evidence from clinical trials that cell replacement in human neurological diseases can lead to symptomatic relief [29].

Here, we discuss some general issues relating to the scientific basis of stem cell–based therapies and their prospects in PD, AD, HD and ALS disorders.

15.2. PARKINSON'S DISEASE

Parkinson's disease results from the extensive loss of dopamine (DA) neurons in the substantia nigra [31, 32]. Current treatment options include therapies that propose to increase dopamine levels by providing a dopamine precursor L-dihydroxyphenyl alanine (L-DOPA), but long-term administration of L-DOPA has become increasingly ineffective with PD progression [33]. More recently, surgical deep brain stimulation has been adopted as a successful treatment for PD patients [34].

Contrarily, cellular approaches for PD focus on the cell replacement of lost DA neurons. Since the late 1980s, successful cellular therapies for PD have utilized human fetal ventral midbrain tissue as a source of DA neurons to transplant into the striatum of PD patients with advanced disease [35-38]. However, potential limitation of using fetal tissue for transplantation includes ethical concerns and religious questions, and the ability to obtain adequate amounts of fetal tissues for

treatment. In addition, reports have pointed out that the survival of transplanted fetal mesencephalic cells in the patients' brain was very low [39]. To circumvent these difficulties, ESCs, iPSCs, MSCs or NSCs have been utilized for large-scale generation of neurons with DA phenotype as a practical and effective alternative for transplantation.

Although there are also considerable safety concerns for ESCs related to their potential for tumor formation or neural overgrowth, previous studies have described that DA neurons derived from ESCs have shown efficacy in PD animal models. DA neurons generated from mouse ESCs after treatment with specific mitogens and signaling molecules such as fibroblast growth factor 8 (FGF8) and sonic hedgehog, [40, 41], have shown electrophysiological and behavioral properties expected of neurons from the midbrain. Additionally, the modulation of Nurr1 [42, 43] or Bcl-XL [44] expression in murine ESCs or co-culture with a mouse bone marrow stromal cell line [45] induces midbrain dopaminergic neurons. In a very recent study, transgenic mouse ESCs line induced at the middle stage (Nurr1 positive cells) of DA differentiation was particularly suitable for grafting since it had the greatest amount of DA neuron survival and behavioral improvement in parkinsonian mice [46].

Interestingly, the striatum transplantation of DA neurons generated from monkey ESCs attenuated neurological symptoms in a primate model for PD [47]. DA neurons were also generated from undifferentiated human NSCs derived from fetal brain and induced behavioral improvement in parkinsonian monkeys [48]. Recently, studies have reported that functional human DA neurons can efficiently be engrafted in animal models of Parkinson's inducing clear behavior recovery [49-51].

Diverse evidence has shown that the transplantation of NSCs cultured *in vitro* into brain damage areas may be an ideal vehicle for cell replacement and production of neurotrophic factors to protect injured neurons and/or to stimulate neuronal growth in patients with neurological diseases [2, 52]. Distinct research groups have described that immortalized hNSC generated from fetal human brain cells infected with a retroviral vector encoding v-myc oncogene shows multipotent differentiation properties, due their capacity of neural differentiation *in vitro* and *in vivo* [52, 53]. Indeed, both immortalized human and mouse NSC lines, HB1.F3

and C17.2 respectively, transduced with tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GTPCH1) genes, for production of L-DOPA, induced functional improvement in a rat model of PD following transplantation into the striatum [54-56].

Another approach based on the use of patient-specific DA neurons derived from iPSCs may provide an ideal cellular source for transplantation therapy for PD since it would eliminate ethical concerns associated with ESCs and their progeny and would avoid immune reactions. However, before application can be considered in patients, the production, growth and functionality of the DA neurons derived from iPSCs and also the potential risk of teratoma formation *in vivo* should be determined [57, 58].

A study has reported that DA neurons generated from iPSCs derived from fibroblasts functionally integrated in the host brain and were able to improve behavior in a rat model of PD upon transplantation into the adult brain [59]. Strikingly, a study that directly compared the differentiation and cellular properties of human iPSCs generated by different reprogramming methods, virus- and protein-based, showed that DA neurons derived from protein-based are functional, and when transplanted into striatum, significantly rescued motor deficits in a rodent PD model [60]. More recently, the combination of five transcriptional factors Mash1, Ngn2, Sox2, Nurr1, and Pitx3 can directly and effectively reprogram human fibroblasts into DA neuron-like cells. They also showed properties of DA uptake and production, exhibited DA neuron-specific electrophysiological profiles and provided symptomatic relief in a rat PD model [28].

Recent advances in deriving iPSCs from patients offer new possibilities for biomedical research and clinical applications for autologous transplantation. The generation of iPSCs from patients with PD has been described in three reports [61-63]. Patient-derived iPSCs from individuals with sporadic PD were differentiated into dopaminergic neurons but failed to show an obvious difference in phenotype compared to control cells [61]. Some evidence has shown that fibroblasts from genetic PD (mutations in the PINK1 gene) can be reprogrammed and differentiated into dopaminergic neurons, indicating that mutation did not affect the ability of patient fibroblasts to be induced into iPSCs [62]. Thus, this

approach could be used to investigate the function of endogenous mutations and for further studies of PD pathogenesis [62]. In another study, DA neurons generated from iPSCs from PD patients could be transplanted into the PD animal model and survive in high numbers, showing arborization and mediating functional effects [63].

Although studies maintain cellular replacement based on DA neurons derived from iPSCs [64] or DA neurons directly converted from fibroblasts [28] as a viable approach for treating PD, environmental enrichment may also support existing DA neurons to prevent further degeneration. Neurotrophic factor-based therapy through direct delivery or viral-based systems demonstrated the potential for excellent ameliorative properties in PD [65, 66]. Remarkably, transplantation of MSCs or NPCs, genetically modified to produce growth factors such as BDNF, VEGF, GDNF, and IGF-I, protect both dopamine neurons and striatal neurons undergoing degeneration in rodent models of Parkinson's [67-73]. Thus, both cellular replacement and environmental enrichment present important consequences to improve efficacy for PD therapy [3].

15.3. ALZHEIMER'S DISEASE

Alzheimer's disease (AD), the most frequent form of dementia, is characterized by degeneration and loss of neurons and synapses throughout the brain, memory loss and cognitive decline [74]. The brain pathology in AD is characterized by intracellular neurofibrillary tangles (composed by phosphorylated tau protein) and extracellular deposition of plaques composed of amyloid β (Aβ) peptides, which represents a proteolytic cleavage product of larger amyloid precursor protein (APP) [74-76].

Based on the amyloid cascade hypothesis, various ''anti-amyloid drugs'' targeting different pathways of Aβ production and/or aggregation have been developed and tested in clinical trials with AD patients. In general, these candidates have so far failed to produce the expected therapeutic breakthroughs. However, proteinases such as neprilysin [77]; insulin degrading enzyme [78, 79]; cathepsin B [80] and plasmin [81] were successfully used as therapeutic agents to reduce Aβ levels in the AD brain. Indeed, intracerebrally injected fibroblasts overexpressing human

neprilysin revealed robust clearance of amyloid plaques at the site of engraftment in the brain of Aβ transgenic mice [82].

In this context, MSCs present a promising therapeutic vehicle to reduce Aβ deposits in AD patients. Intracerebral transplantation of BM-MSCs into double-transgenic mice (APP/PS1), a model of age-dependent AD, promoted microglial activation, rescued cognitive impairment, and reduced Aβ and tau pathology in the AD brain [83, 84]. Together, these results revealed that BM-MSCs, by unknown mechanism(s), are able to modulate microglial cells which in turn mediate Aβ reduction and rescue AD-like pathology [20]. Furthermore, *in vitro* co-culture of primary hippocampal neurons with MSCs from umbilical cord blood reduced the apoptosis induced by Aβ**.** Furthermore, in a mouse AD model, MSCs treatment causes restoration of learning/memory function [85].

Previous studies have described that nerve growth factor (NGF) is required to prevent the degeneration of cholinergic neurons and to enhance memory in AD-like animal models [86-89]. Indeed, a phase I clinical trial of *ex vivo* NGF gene delivery was performed in individuals with mild Alzheimer's disease, implanting autologous fibroblasts genetically modified to express human NGF into the forebrain and the study analysis suggested improvement in the rate of cognitive decline [90]. In a chronic AD animal model, NSCs, stably transduced with hNGF, was engrafted into the cerebral cortex and presented a significant improvement in learning and memory function [91]. In other studies, the transplantation of mouse ESC-derived NPCs improves cognitive function in rat models of Alzheimer [92].

Therapeutic delivery of brain derived neurotrophic factor (BDNF) has also been explored as a promising candidate for Alzheimer's disease [93]. Aged transgenic mice that express pathogenic forms of amyloid precursor protein, presenilin, and tau presented an enhancement in cognitive function after brain transplantation with mouse NSCs expressing BDNF [94].

Remarkably, a range of recent studies have used NSCs as a valuable source of cells for cell replacement and gene transfer for AD therapy due to their migratory capacity after brain transplantation [95, 96]. In fact, iPSCs have not yet been used in AD therapy models. However, recently, primary fibroblasts from patients with familial

Alzheimer's disease have been reprogrammed and differentiated in functional neuronal cells, providing a human cell-based model of AD that would be crucial for drug discovery as well as for investigating mechanisms of the disease [97].

15.4. HUNTINGTON'S DISEASE

Huntington's disease is an autosomal dominant polyglutamine disorder characterized by the accumulation of the nucleotides CAG in the huntingtin gene. This disease causes cellular dysfunction and loss at numerous CNS sites including the striatum. HD manifests with involuntary motor activity, cognitive impairment and emotional disturbances. Despite the known genetic basis for HD, the mechanisms involved in the pathogenesis of HD remain essentially unknown and this impedes effective therapeutic interventions. A major motivation for research into the treatment of HD has centered on reparative strategies using cell replacement, manipulation of endogenous stem cells and/or neurogenesis, or trophic factor administration on the striatum [98].

A pioneer study of a human cellular therapy trial using fetal neural stem cells allografts showed motor and cognitive improvement in HD patients [99]. Earlier studies in HD animals models reported that fetal striatal grafts improve motor and cognitive dysfunction [100]. Interestingly, striatal grafts survived with apparent integration in the striatum of a transgenic mouse model of HD, which in turn presented modest behavioral effects after surgery [101]. The latter study is consistent with a clinical trial that demonstrates that grafts from human fetal striatal tissue in HD patients can survive and differentiate, and are unaffected by the disease process [102]. In addition, transplantation of striatal grafts of human fetal stem cells elicits behavioral and anatomical recovery in a rodent model of HD [103]. Additionally, human ESC-derived neural precursors can lead to a behavioral recovery, as well as neuronal differentiation, in the pre-clinical model of HD [104].

Cell therapies in HD patients using human fetal-derived cells have shown clinical success. Nonetheless, a recent study has reported a graft overgrowth, composed by neurons and glia, in a HD patient who received fetal neural transplantation and reminds researchers of the potential risk of mass lesion development with this procedure [105]. The use of fetal-derived striatal cells for transplantation into the

degenerative adult brain is safe and partially effective in terms of functional response, although ethical and practical considerations in terms of cell source of human fetus-derived allografts should be regarded. Thus, alternative strategies for cell therapy using NSCs in HD have been initiated [106] in animal models of HD, although still only a few studies have demonstrated a functional outcome.

The implantation of NSCs into striatum of an HD rat model of prior to lesion formation demonstrated significantly improved motor performance and increased resistance to striatal neuron damage compared with control [107]. This result indicates that early intervention using cell transplantation could be effective in pre-clinical HD patients carrying the mutant HD gene. Thus, functional improvements confirmed by isolated cell types provide similar functional benefits to those observed with fetal tissue, although mechanisms of cellular therapy protection were not examined.

In another study, the transplantation of autologous bone marrow stem cells in the damaged striatum of a rat HD model significantly reduced working memory deficits [108], suggesting that growth factors could be released by transplants allowing cell surviving. To address the role of environmental enrichment in cellular therapy for HD, NSCs/NPCs and MSCs have been genetically modified to overexpress and release neuroprotective trophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CTNF) and glia cell-derived neurotrophic factor (GDNF) [109].

In an earlier transplantation study, genetically modified NSCs producing NGF or BDNF showed a protective effect of the neostriatum against excitotoxic damage [110]. NPCs expressing GDNF, transplanted into HD rodents, protected neurons and promoted functional recovery [67, 111]. In a recent study and according with NPCc data, MSCs overexpressed BDNF had also significant ameliorative effects on disease progression in a HD mouse model [112]. Collectively, the use of stem cells engineered to overexpress a range of neurotrophic factors in transplantation studies in HD models confers benefits reducing disease progression.

Although recent evidence has demonstrated improvements in motor and cognitive functions observed in HD animal models following stem cell transplantation,

further studies are now required to address relevant questions regarding the availability and safety of stem cells for clinical trials.

15.5. AMYOTROPHIC LATERAL SCLEROSIS (ALS)

Amyotrophic lateral sclerosis (ALS), is a relentlessly neurodegenerative disorder characterized by degeneration and loss of motor neurons in the cerebral cortex, brain stem and spinal cord, leading to loss of coordination and muscle strength. Multiple pathogenetic mechanisms are likely involved in ALS, which makes the development of conventional drug therapies difficult [113]. To date there is no effective treatment for ALS patients and stem cells represent a new therapeutic approach offering both cellular replacement and trophic support on motor neuron survival and function.

NPCs are normally produced in the CNS in response to the loss of motor neuron in ALS [114], but to date, endogenous NPC populations have proven insufficient to reverse the disease condition in ALS [114]. However, several studies have tested the capacity of exogenous stem cells transplanted into the lumbar spinal cord to rescue ALS animal models. There is increasing evidence that it is possible to generate functional motor neurons in culture from stem cells (ESCs and NPCs) able to populate the embryonic spinal cord, extend axons, and form synapses with target muscles [27, 115, 116]. The incorporation of NSCs, isolated from SVZ of the adult mice and differentiated into cholinergic neurons, into animal ALS spinal cords delayed the onset of the disease [117, 118]. Human embryonic germ cells transplanted into the cerebrospinal fluid of rats with motor neuron injury migrated into the spinal cord restoring neurologic function *via* enhancement of host neuron survival and function [119]. NSCs grafts isolated from human fetal spinal cord were also effective in delaying the onset and disease progression in a mouse ALS model since these cells integrate into the diseased spinal cord and establish synaptic connections with host neurons, one of the most fundamental requirements for motor neuron replacement [120]. Recent evidence showed that human spinal cord NSCs derived from human fetus were transplanted into the spinal cord of rats in an ALS model, and the neurological function of NSC-transplanted animals was well preserved, but disease onset was not different from the untreated controls and the overall animal survival was also not affected [121].

Interestingly, results of an ongoing phase I trial of intraspinal injections of fetal-derived neural stems cells in ALS-patients showed that the treatment has no surgical complications and patients' clinical status was stable after transplantation with no evidence of disease progression [122]. A previous study described that iPSCs isolated from an ALS patient [123] that possess properties of embryonic stem cells, which were successfully directed to differentiate into motor neurons, could be an ideal cellular source for screening new drug candidates [123]. The potential of growth factors to mediate neuroprotection on motor neurons in ALS models has been investigated and three methods for growth factor delivery have been used: direct application, viral delivery and stem cell-based delivery. Several studies report stem cell production of growth factors including GDNF, BDNF, IGF, and VEGF [117, 120, 124]. These studies demonstrated that NPCs secreting growth factors integrate normally into the spinal cord, survive, differentiate and provide long-term production of growth factors, which support neuroprotection for existing neurons.

Several groups have demonstrated that intraparenchymal delivery of hMSCs is safe and can delay loss of motor neurons in ALS mouse model [125, 126]. hNPCs, modified to secrete GDNF, survived and improved maintenance of lumbar spinal cord neurons of ALS rodents model [127, 128].

A human cellular therapy trial has already demonstrated progress in ALS treatment by intraspinal cord injection of MSCs. Autologous transplantation of bone marrow-derived MSCs into the thoracic spinal cord showed no significant acute or late side effects and four of the patients showed significant slowing of the linear decline of forced vital capacity [129]. In fact, the development of new stem cell lines is required in attempt to expand our understanding about the potential use of stem cells in ALS.

CONCLUSION AND FUTURE PERSPECTIVE

The development of cell-based therapies for neurodegenerative diseases that currently lack effective treatment is still at an early stage. However, as we have shown in this chapter, considerable progress has been made in this direction and a continuous improvement in developing approaches to generate distinct type of

neurons from human stem cells, for cell replacement therapy in neurodegenerative disorders, is needed. Strategies used to develop stem-cell based therapies for neurodegenerative diseases discussed in this chapter are illustrated in Fig. **1**. Herein, we have shown that findings generated in the laboratory are now slowly being translated into timid clinical trials that have aimed either at cell replacement or at neural tissue delivery of therapeutic molecules using stem cells as a carrier. Still, many basic issues remain to be solved and mechanisms which regulate the proliferation, migration, differentiation survival and function of stem cells and their derivatives need to be elucidated. Despite the many challenges for cell therapy lying ahead, we are still optimistic that stem cells have great potential to cure human neurological diseases.

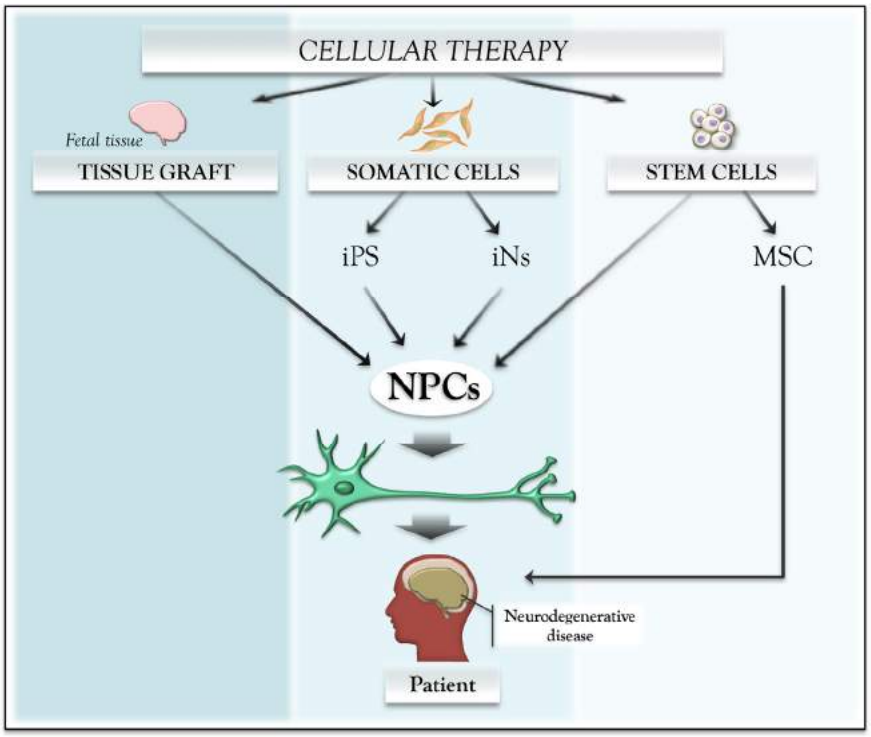

Figure 1: Cellular therapy for neurodegenerative diseases. Different sources of cells give rise to neural precursor cells (NPCs), the most common stem cell source to get neurons for neurodegenerative disorders cellular therapy. NPCs can be provided directly by fetal tissue graft or indirectly by somatic and stem cell differentiation. Genetic reprogramming of somatic cells originates induced neuronal (iN) cells directly or induced pluripotent stem cells (iPS) that can be differentiated into NPCs. Another class of stem cell, mesenchymal stem cells (MSCs), can be directly used in patient treatment.

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CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

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