CHAPTER 12

Genes, Aging, and Parkinson's Disease

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12.1 Introduction

Aging is characterized by a progressive deterioration of physiological functions and concomitant increased risk of morbidity and mortality. Aging constitutes a major risk factor for most complex diseases, including neurodegenerative disorders and Parkinson's disease (PD). In advanced economies, lifespan extension has increased dramatically, the population is rapidly aging, and in the near future healthcare and general costs for the growing proportion of elders will substantially impact society. Unraveling the mechanisms promoting healthy aging to devise effective intervention to delay or avoid the onset of age-relate pathologies therefore constitutes a matter of primary social and economic importance.

The timing and the dynamics of lifespan increase observed during the last century suggest that the principal factors responsible for such improvement are not genetic (*i.e.* not caused by acquisition of advantageous genetic variants) because the pace of lifespan extension has been too rapid to be compatible with the evolutionary timescale. Nonetheless, multiple elements indicate

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that genes influence lifespan and understanding the extent of genetic contribution is fundamental to decipher this phenomenon and to identify potential targets to mitigate the noxious effects of aging.

A discussion on genetics of aging cannot ignore the fundamental problem posed by the heterogeneity of the aging phenotype. While a long lifespan is a well-recognizable trait, healthy aging is definitively more complex and can be defined in several ways, such as the preservation of certain functions such as cognition or the absence of morbidity in advanced stages of life. Decoding the genetic landscape favoring healthy aging is therefore challenging because of the extreme heterogeneity in the phenotype.

The exact role of aging on the etiopathogenesis of PD and of other neurodegenerative disorders is still debated. There is consensus, however, that promoting successful aging, which is associated with a desirable general health status and, eventually, extended lifespan, would be highly desirable in view of the aging role as a main risk factor for complex diseases. An overall good health status and longevity are to some extent correlated because it has been observed that long-lived individuals tend to be healthy for most of their lives. Longitudinal studies following the same cohort of cases in time, in fact, have shown that a lower presence in midlife of risk factor for conditions such as cardiovascular diseases predicts longer and healthier lifespan.[1–4] The progression rate and the severity of aging are influenced by complex interactions between genes and environment, including lifestyle. In this section we will analyze the genetic and environmental factors so far known to impact aging and discuss their relevance for the control of cellular oxido–reductive homeostasis and PD.

12.1.1 Do Genes Influence Lifespan?

The aging phenotype is heterogeneous and is produced by the concomitant action of numerous biological factors. It is therefore extremely challenging to determine the features of genetic landscapes favoring longevity and/or healthy aging.

A first level of information on aging genetics can be inferred by epidemiological studies designed to determine whether a prominent trait of aging, for instance longevity, is inheritable and, if so, to what extent. Here, the influence of genes on aging emerges from several investigations that approached the problem from different perspectives. Analyses in large cohorts of twins demonstrated that the lifespan of homozygous pairs was more similar than that observed for same-sex heterozygous siblings[5,6] and these investigations estimated longevity heritability to be around 25%. Investigations in cohorts from the general population (*i.e.* population-based studies) led to results that are comparable with those gathered in twins. For instance, studies on centenarians have shown that siblings and offspring of exceptionally long-lived individuals have higher chances to live longer than average;[7–10] here, the contribution to heritability is slightly lower than that observed in twins and has been estimated to be around 20%.

When looking at these figures, however, it should be considered that the followed phenotype – longevity – is multi-dimensional because it derives from a combination of factors; healthy aging, for instance, may be promoted by favorable environmental and societal conditions. Therefore, selecting a population exclusively on the basis of its members' lifespan can lead to loss of information about potential genetic information because of confounding factors. Additional investigative strategies following alternative and more specific aging-related phenotypes, which in turn contribute to longevity, might provide better insights into the effects of genes on aging. Here, examples come from studies monitoring traits such as weakness, defined as handgrip strength, or lower extremity function display much higher degrees of heritability.[11,12] Overall, these researches provided preliminary evidence that the duration of life may be influenced by genetic factors. Although it has been suggested that genetic influence of longevity varies among different ethnicities,[13] further investigations are required to properly address this issue, which is intrinsically biased by the different life-styles among these groups as well as inequalities in their socioeconomic conditions and life expectancy.[14]

12.1.2 Which Genes Influence Lifespan?

Provided that epidemiological studies ascertained the influence of genes on aging, the next logical step would be to identify the specific genes underlying lifespan extension or early aging. Traditionally, these studies are performed through linkage analysis, in which statistical methods are used to determine the association between genetic markers (*e.g.* a certain genomic region) and a specific trait. In its more sophisticated form, linkage analysis involves genome-wide genotyping for single nucleotide polymorphisms (SNPs) using microarray technology[15] or next-generation DNA sequencing[16] (genome-wide association studies or GWAS). Linkage studies, however, present some intrinsic difficulties: sample size is often an issue, as very large populations are required to reach adequate power and, seeking association with a heterogeneous, multi-component trait such as longevity, only aggravates the problem.[17] Linkage studies, in fact, are rather suitable to detect variants that exert strong effects on the studied trait. Because general wisdom assumes that genetically human aging is produced by a combination of multiple *loci*, each with modest effect, linkage studies seem to be further penalized in this specific field. Genotyping of SNPs implies additional challenges as SNPs number in the human genome is of the order of 10^6 and might even exceed 10^7.[18,19] Under these circumstances, association studies on aging have traditionally suffered from a significant proportion of false-positive data. Careful validation, possibly through meta-analysis of pooled data from different studies, is imperative.[20]

On the basis of these technical and conceptual hurdles, it is not surprising that the results from linkage studies to discover the genetic basis of longevity failed to meet expectations. More encouraging results have been obtained in

studies exploring association between a specific aging trait and candidate genes, in cases and control groups. In contrast to genome-wide studies, this approach focuses on *loci* that, on the basis of previous biological knowledge (*e.g.* derived from animal models), constitute presumptive candidates to influence aging. Because the strategy is circumscribed to determined *loci*, it has more power than linkage analysis to detect variants with modest effects. It should, however, be noted that case–control studies focused on aging involve the comparison between elderly and young cohorts and that this design poses distinctive methodological challenges concerning selection of appropriate controls groups. For instance, an insidious assumption is that the frequency of the alleles influencing lifespan is equal in the two initial populations of young and elders, or, in other words, that the populations of young and elders are not stratified. The frequency of the studied allele, therefore, should be comparable in a cohort of centenarians born in the first decade of the twentieth century (assuming that the study is performed in the second decade of the twenty-first century) and in a group of putative controls in their seventies, born in the third decade of the same century. This supposition, however, may be inaccurate because of several factors. Migration fluxes in the considered population, for instance, may change significantly the frequency of a certain allele and – because of substantial geographic mobility in certain periods of the past century – two populations born only a few decades apart may exhibit significant difference in allelic frequencies. Obviously, genetic studies are designed to distinguish between different ethnic groups. It should be also noted, however, that such distinction rarely occurs within the Caucasians ethnicity and thus groups such as Germans, English, and Italians are treated as one, despite plausible differences in allelic frequencies. The issue of initial allelic frequency of genes eventually influencing lifespan constitutes a serious confounding factor leading to spurious results. An excellent article by Lewis and Brunner[21] provides additional details on this and other biases undermining association studies comparing young controls and elder cases, and also invites caution when considering and interpreting data from this kind of analysis.

Given the complexity and the challenges of genetic studies on longevity, it is not surprising that an exiguous number of *loci* associated with extended lifespan have been identified thus far. Although several reports have indicated numerous genes that, in principle, might be associated with longevity, most of these candidates have not been confirmed in subsequent independent investigations and therefore their relevance for longevity remains unclear. In fact, only two genes have been consistently associated with longevity in multiple studies: Apolipoprotein E (*APOE*) and the Forkhead-box O (*FOXO*) transcription factor 3A (*FOXO3A*),[22–24] also reviewed in ref. 25,26. Importantly, contribution of these genes to longevity has been recently confirmed in a large multi-center study (the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium) that performed meta-analysis of data from a very large population of longevity cases and relative controls.[27] Given the study's considerable sample size, which is generally regarded as the

critical ingredient for successful GWAS, it is intriguing that no genes other than *APOE* or *FOXO3A* emerged as candidates with significant association. The authors ascribe such an outcome to the intrinsic heterogeneity of longevity as a trait and the involvement of rare allelic variants; alternatively, regulation of longevity might depend upon different combinations of genetic variants, which would have modest effects individually, but interact synergistically when simultaneously present in a genotype. In all such cases, associations would not be captured by GWAS.

In the present chapter, we will focus the discussion on APOE or FOXO3A, also highlighting their relevance for PD and redox homeostasis; for those *loci* with uncertain association, the reader should refer to other excellent reviews.[17,25,26]

12.2 Apolipoprotein E

Apolipoprotein E (APOE) is a 34 kDa protein involved in lipid transport and delivery between the different tissues of the organism.[28] Although its function is not limited to the central nervous system (CNS), for the purposes of this chapter we will emphasize its role in the brain, referring the reader to other excellent reviews for a more general discussion (*e.g.* ref. 28). A seminal and classic article by Brown and Goldstein, who pioneered studies on receptor-mediated cholesterol transport, still represent an excellent overview of this biological process and is highly recommended for further reading.[29] APOE is particularly important in cholesterol transport and reuptake from the blood and it was originally described as a component of the very low density lipoproteins (VLDL) and the chilomicrons. These particles are spherical macromolecular complexes instrumental in transporting hydrophobic lipids otherwise insoluble in the bloodstream. The designation VDLD derives from the initial characterization of this family of particles on the basis of their density inferred by ultracentrifugation analysis.[30] Structurally, lipoproteins consist of a lipidic core wrapped in a phospholipid monolayer, which in turn embeds the apolipoproteins. The lipidic core, essentially a lipid droplet, is composed by triacylglycerols and cholesterol, and the latter is esterified in its single polar hydroxyl residue to further ease solubilization by the phospholipid monolayer and therefore transport. Humans express at least ten apolipoproteins, including APOE, which differ in their molecular features and functions.[30]

In the brain, APOE is primarily synthesized in astrocytes[31] – and to a much lesser extent by microglia[32] – and is essential for glial-mediated delivery of cholesterol to neurons. Cell-culture experiments indicate that APOE is secreted by astrocytes as a lipoprotein complex of discoidal shape, containing little lipid. *In vivo*, however, in samples from human cerebrospinal fluid (CSF), APOE complexes have been reported to form spherical particles containing esterified cholesterol in its core.[33] Whether this discrepancy is due to artifacts in cell-culture condition or rather reflects a maturation process of the particle from secretion to release into the CSF, which would also lead to enrichment

of the lipid core, is unclear. It is, however, ascertained that association with lipids is vital for APOE stability as demonstrated by the sharp reduction in its levels in the brain of mice lacking the ATP-binding cassette, sub-family A, member 1(Abca1) enzyme, which specifically lipidates APOE.[34,35]

Despite the fact that neurons can autonomously produce cholesterol, glial supply remains crucial to generate and maintain synaptic connections.[36–38] APOE is the principal apolipoprotein in the brain as also evidenced in studies on knockout mice, which show reduced brain functional connectivity as determined by resting-state functional MRI, decreased immunoreactivity for the post-synaptic marker PSD-05, and reduced cerebral blood flow.[39]

APOE is a polymorphic gene encoding for a protein regulating lipid homeostasis. In humans, APOE exists in three allelic forms at a single gene locus on chromosome 19, which are denominated APOE2, APOE3, and APOE4 (also known as ε2, ε3, ε4)[40] with respective frequency of 8.4%, 77.9%, and 13.7% in the global population. The assortment of these alleles in the genotype exerts a moderate impact on both common diseases and longevity. The ε4 allele is in fact a moderate risk factor for Alzheimer's disease (AD) and cardiovascular diseases[28] and is also significantly less presented in the genotype of long-lived individuals, and in centenarians in particular.[22,23] On the contrary, the ε2 allele might exert a protective function against AD and cardiovascular disorders and is enriched in centenarians.[22,23] The precise reasons underlying these effects are poorly understood. It is, however, clear that the polymorphisms in ε2, ε3, and ε4 modify basic biological properties of the respectively coded proteins: APOE2, APOE3, and APOE4.

12.2.1 LDL Receptors

The mechanism used by apolipoproteins to mediate lipid internalization is receptor-mediated endocytosis. In mammals, the LDL receptor (LDLR) family encompasses at least seven different genes and APOE can bind to most, if not all of its members.[41,42] Some members, for instance LRP1 and megalin, are essential for the proper development of the nervous system, as evidenced by embryonic lethality of knock-out mice.[43,44] The receptor–ligand complex is internalized in clathrin-coated endocytic vesicles that eventually fuse with lysosomes, whose enzymes hydrolyze the esterified cholesterol allowing its release in the cytosol. The LDLR receptor escapes degradation and is recycled on the cell surface to participate to the next LDL uptake cycle.[42] Importantly, LDLR function is not limited to vesicle transport *via* endocytosis as they also actively participate to cellular signal transduction,[42] which is mediated by specific interactors with diverse functions binding to the cytosolic domain of the receptor. On the basis of actual knowledge, the majority of genes encoding for LDLR interactors mediating signalling lack elements that might link them to PD or even to dopaminergic (DAergic) neurons function. In some cases, which are described in the next paragraphs, functional evidence might suggest potential involvement. Data in support of this conjecture, however, are lacking thus far.

The LDLR interacting protein Disabled-1 (DAB1) constitutes the intracellular effector of Reelin (RLN), an extracellular matrix glycoprotein essential for neuronal migration and positioning during development, which activates a pathway that is also relevant for the development of DAergic neurons.[45,46] The pathway is also important in the adult brain and its deregulation has been implicated in AD and Creutzfeld–Jacob disease.[47–49] RLN interferes with aggregation of amyloid-beta[50] and accumulates in amyloid-like deposits during normal aging.[51] No reports describe investigations on the role of the RLN/DAB1 in PD thus far. RLN involvement in DAergic neuron development and its abnormalities in normal aging, however, might indicate that this pathway could also be modified in PD.

Post-synaptic density protein 95 (PSD-95) is a scaffolding protein that stabilizes glutamate receptor, and *N*-methyl-D-aspartic acid (NMDA) receptors in particular in synapses. More recently it has been shown that PSD-95 also interacts with D1 and D2 dopamine (DA) receptors and, accordingly, mice with targeted deletion of PSD-95 show motor impairments, striatal degeneration, and altered DA-glutamate interplay.[52] Additionally, PSD-95 modulates D1 dopamine receptor trafficking and influences L-DOPA dyskinesia, which is the major undesired side effect in DA replacing palliative therapy in PD.[53]

CAPON regulates neuronal nitric oxide synthase (nNOS),[54] which has been implicated in neurodegeneration in some PD models.[55,56] Additionally, CAPON might participate in iron (Fe) homeostasis regulation,[57] which is severely deranged in the *substantia nigra pars compacta* (SNpc) of PD patients.[58]

A further nexus between LDLR and PD might derive from the ability of its members, and of LRP1 in particular, to bind proteins that might have an implication in the disease such as lactoferrin[59–61] and alpha2-macroglobulin.[62]

12.2.2 Effects of Polymorphisms on APOE Function

APOE isoforms present primary sequence differences at the level of amino-acidic residues 112, 158, or both. Both sites can present either a cysteine (Cys) or an arginine (Arg) residue that are differently assorted in the isoforms: Cys112, Cys158 in APOE2, Cys112, Arg158 in APOE3, Arg112, Arg158 in APOE4. The mechanistic details underling the detrimental effects of the E4 allele are dimly understood, yet it is clear that these substitutions alter ApoE structure and biological properties, as reviewed by Zhong and Weisgraber.[63]

Amino acid substitutions in APOE3 and APOE4 directly influence the structural properties of the protein. In its mutant forms, APOE better succeeds in maintaining relatively stable equilibrium states that are intermediates between the unfolded and the native configuration. These folding intermediates – called molten globules – exhibit distinctive properties different from those of the protein in its native state, which might result in detrimental consequences.[64] For instance, in the molten globule state APOE4 exposes otherwise buried proteolytic sites[65] and is therefore more susceptible to proteolysis. A higher prevalence of molten globules, which occurs by virtue

of their increased stability in APOE3 and APOE4 variants, might therefore underlie detrimental processes in genotypes carrying these polymorphisms. Additional conformational changes concern the tertiary structure of the native protein state; an Arg substitution at residue 112 leads to a conformational shift that allows a novel interaction between Arg61 and glutamate (Glu)255, which compacts the protein conformation. Conversely, when a Cys is present at position 112, Arg61 is shielded by two helices in the structure and is therefore not available for polar interactions with Glu255. Cysteine to Arg substitution at position 158 sharply reduces LDL-receptor binding activity. The same does not occur, however, for the Cys to Arg substitution at residue 112. It is also unknown if the detrimental consequences associated with APOE3 and APOE4 isoforms, which both carry an Arg at residue 158, are related to the low LDL-receptor binding activity.[66]

Furthermore, substitutions in APOE variants also alter the protein stability, particularly at its N-terminal, and render APOE4 less stable than APOE3, which is in turn less stable than APOE2. Instability has important pathogenic implications because it renders the isoform more prone to aggregation, at least *in vitro*.[67,68] ApoE can also promote aggregation of other proteins, most notably amyloid-beta[69] and in patients APOE4 expression levels correlate with the extent of amyloid deposition.[70]

Cysteine residues in positions 112 and 158 can engage in disulfide bonds forming homodimers with reduced LDL-receptor binding activity. Homodimers, obviously, cannot form in the APOE4 form where Cys are substituted. A significant portion of APOE2 and APOE3 circulating in plasma is in the form of homodimers (about 50%),[71] which constitute an even larger proportion in the CSF.[72] There are only limited studies investigating how oxidative stress intrinsic to aging or neurodegenerative diseases impacts Cys residues of APOE and the eventual functional consequences. *S*-nitros(yl)ation of APOE2 and APOE3 after interaction with NOS1 has been recently described.[73] Studies on other modifications such as *S*-glutathionylation or the formation irreversible oxidative modifications (*i.e.* sulfenic, sulfinic, or sulfonic acids) have not been performed thus far, at least to the best of our knowledge.

12.2.3 APOE and Parkinson's Disease

Traditionally, APOE polymorphisms have been associated with higher risk of AD. However, more recent evidence indicates that APOE may also be relevant for PD, and particularly for some aspect of its pathology. Genetic case-control association studies have shown that APOE4 increases the risk of dementia in synucleopathies (*i.e.* in PD, PD with dementia, and dementia with Lewy bodies).[74] Additionally, an exhaustive, multi-centric study on a relatively large cohort of PD case revealed that, in patients suffering with dementia, APOE4 allele is associated with more severe deficit in multiple cognitive domains.[75] Interestingly, in PD patients without dementia, APOE4 association was limited to the cognitive domain of semantic verbal fluency, whose deterioration is typical of early AD rather than PD and is rather is attributable

to the derangement of the temporal cortex. This evidence suggests that APOE influences processes that are predominant in AD pathology, but are to some extent shared also by PD. It should also be emphasized, however, that the APOE genotype did not correlate with measures of AD neuropathological changes in studies on PD autopsy specimens[76] and thus additional studies will be necessary to fully understand whether the APOE4 genotype effectively increases the load of AD-like neuropathological changes in PD. The molecular mechanisms underlying APOE effects in PD are obscure. The process seems to be unrelated to amyloid-beta processing because, in PD patients, the APOE genotype did not correlate with the brain levels of amyloid-beta.[77] Increase in both APOE and LRP1 expression has been observed in neuromelanin (NM) containing DAergic neurons of the SNpc[78] and led to the hypothesis that perturbation in lipid metabolism might participate to deterioration. APOE cascade is also activated in PD animal models, as demonstrated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice.[79]

12.2.4 APOE Oxidative Stress

APOE genotype also influences redox tolerance and homeostasis, which are central to PD etiopathogenesis.[80–82] APOE can protect cells from hydrogen peroxide (H_2O_2) toxicity, it can scavenge reactive oxygen species (ROS), and it can restrain copper (Cu)-catalyzed lipoprotein oxidation,[83] as reviewed in ref. 84. These properties are enhanced in E2 and E3 isoforms, when compared with E4 (E2>E3>E4). Accordingly, AD patients carrying the E4 polymorphism exhibit signs of redox imbalance such as lipid peroxidation in the brain[85] and increased ROS in the bloodstream.[86,87] APOE also protects synaptosomes from amyloid-beta-induced oxidation in an allele-dependent way (E2>E3>E4).[88,89] Additionally, APOE interferes with the metabolism of metals. Initial experiments used metal-chelate affinity chromatography to demonstrate APOE capacity to interact with different metal cations and determined that the protein has strong affinity for Cu, and to a lesser extent, for ferric (Fe^{3+}), ferrous (Fe^{2+}), and zinc (Zn) cations.[83] The exact binding metal site remains to be identified, but it has been speculated that the four-helix bundle at the protein N-terminus is structurally compatible with metal coordination.[83] The latter, however, does not differ among the different isoforms.[83] Whether these factors participate to PD etiopathogenesis is unknown, but it is tempting to speculate that APOE genotype might contribute to perturbation of Fe metabolism, which is central to PD pathogenesis.[58]

12.3 FOXO3A and FOXO Family

FOXO3A belongs to the sub-family of the FOXO transcription factors, which are homologs of *C. elegans* DAF-16.[90,91] Members of the FOXO family are highly conserved, especially at the level of the 110-amino acid DNA binding domain, and are found in species ranging from yeast to humans. FOXOs play a central role in multiple biological processes, including cell cycle and

differentiation,[90,91] DNA-damage repair,[92,93] metabolism,[94–96] apoptosis,[97,98] oxidative stress resistance,[99,100] and longevity.[101–104] Four members of the FOXO family, FOXO1A, FOXO3A, FOXO4 and FOXO6 have previously been described[105] in mammals and are ubiquitously expressed with the exception of FOXO6, which is mainly expressed in the CNS and liver.[96,106,107] They all share the common characteristic of being regulated by two evolutionally conserved signalling pathways: the canonical insulin/IGF1/PI3K and AKT signalling pathways[108–110] negatively regulating FOXOs activity, and the oxidative stress JNK-mediated signalling activating the members of the FOXO family.[111,112] In addition, FOXOs activities are fine tuned by several other signalling pathways that include acetylation, ubiquination, methylation, adenosine monophosphate-activated protein kinase (AMPK) regulation, and thiol oxidation.[112,113]

Because the multiple binding sites located within their structure, FOXOs can function both as transcriptional activators or repressors, probably depending on the range of associated cofactors. These transcriptional factors have overlapping gene expression patterns both during development and in the adult. In addition, FOXO1, FOXO3A and FOXO4 bind to the same DNA target sequence and are capable of regulating the same target genes. FOXOs members show functional redundancy, as demonstrated by deletion studies in mice. Besides FOXO1 null mutation, which induces lethality due to incomplete vascular development, FOXO3A null mutants were found to be viable, although showing hematological abnormalities together with widespread organ inflammation[114] and abnormal ovarian follicular development in females. FOXO4 null mutants show no phenotype.[115]

12.3.1 FOXO3A Biological Functions

As previously mentioned, the members of the FOXO family and FOXO3A in particular, have been involved in a vast range of cellular processes. Several reports have shown that overexpression of the constitutively active nuclear form of FOXO3A induces cell-cycle arrest in the G1 phase by enhancing the expression of the cyclin-dependent kinase inhibitor p27.[116] Expression of the native form of FOXO3A has been shown to directly activate transcription of the p130 gene, which can induce cells to enter in a quiescent state[117] and to directly activate the DNA-damage response *via* the expression of the growth arrest and DNA-damage-inducible protein alpha (*GADD45a*) gene by inducing a delay in the G2-M phase in CCl39 fibroblasts exposed to ultraviolet (UV) irradiation.[118] FOXO3A is also directly involved in apoptosis induction, since it was found to activate the intrinsic apoptotic pathway through the modulation of genes belonging to the B-cell CLL/lymphoma 2 (Bcl-2) family such as Bcl-2-like protein 11 (Bim),[119] p53 upregulated modulator of apoptosis (PUMA),[120] TNF-related apoptosis-inducing ligand (TRAIL),[121] and Fas ligand.[122]

One of the most recent discoveries about FOXO3A activities is related to its significant role in modulation of mitochondria energy metabolism and in the regulation of response to oxidative stress. The effects of FOXO3A

on mitochondrial functions were studied on colon cancer cell lines, where activation of FOXO3A resulted in a substantial down-regulation of mitochondrial genes and changes in the levels of mitochondrial proteins such as mitochondrial import receptor subunit TOM20 homolog (TOM20) and cytochrome C oxidase1 (COX 1). FOXO3A activation also caused a decrease in mitochondrial DNA copy number and a reduction in the mitochondrial respiration activity that was mediated by a FOXO3A-dependent inhibition of c-myc.[123] Controversially, in mouse fibroblasts and skeletal myotubes subjected to glucose restriction, FOXO3A accumulates in mitochondria in an AMPK-dependent manner. Upon sirtuin 3 (SIRT3) activation, FOXO3A mediates mitochondrial polymerase recruitment to DNA with consequent transcription of the core catalytic subunits of the electron transport chain (ETC).[124]

FOXO3A has been revealed to have a prominent role in protecting cells from elevated oxidative stress levels. The activation of FOXO3A *via* the AKT pathway protects quiescent cells from oxidative stress by directly increasing the expression of the manganese superoxide dismutase (MnSOD) gene.[125] As in many transcription factors, reversible acetylation, which increases transactivation by increasing the affinity for the DNA, fine tunes the FOXO3A function.[126] Here, an important role is played by SIRTs, a class of histone deacetylases dependent from nicotinamide adenine dinucleotide (NAD^+) able to suppress transcription on the genome-wide scale.[127] Because they are regulated by NAD^+, SIRTs constitute a functional nexus between metabolism and gene expression regulation and have been implicated in multiple biological processes including the pathogenesis of neurodegenerative disorders and aging.[127,128] SIRT3-dependent activation of FOXO3A increases the expression of MnSOD and catalase (CAT) in different cell types,[129–131] thus increasing cell survival. SIRT3-mediated deacetylation of FOXO3A positively regulates the expression of dynamin-1-like protein (DRP1), mitochondrial fission 1 protein (FIS1) and mitofusin-2 (MFN2) to coordinate mitochondrial fission and fusion in human endothelial cells.[132] It has also been demonstrated that resveratrol, a polyphenolic compound contained in red fruits and wine, mediates its potent antioxidant effect by activating the SIRT3/FOXO3A pathway, which in turn leads to up-regulation of mitochondrial genes such as ATP synthase 6 (ATP6), COX1, cytochrome b (CYTB) and NADH dehydrogenase 2 and 5 (ND2 and ND5) and leads to reduction of mitochondrial ROS reduction production.[128] Reperfusion after hypoxia, a condition leading to massive ROS production, affects cellular localization of FOXO3A by inhibition of the AKT pathway and induction of a rapid nuclear translocation of this transcription factor.[133] Detrimental effects of hypoxia/reperfusion in rat cardiomyocytes can be mitigated by acetylcholine treatment. Mechanistically, this intervention strongly reduces oxidative stress by inducing MnSOD expression through FOXO3A/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) signalling pathway, and up-regulating Cu/Zn superoxide dismutase (CuZnSOD) in the cytoplasm, thus protecting against oxidative injury and cellular apoptosis.[134] Finally, it has been

demonstrated that, in hearts from rat subjected to ischemia/reperfusion, resveratrol administration activates the SIRT3/FOXO3A axis promoting the activation of the PINK1/Parkin pathway potentiating the mitochondrial fission leading to mitophagy in rat cardiomyocites.[135]

12.3.2 FOXO3A and Protein Homeostasis

The role of FOXO members in the regulation of protein homeostasis and protein degradation has been exhaustively studied in muscle tissues and increasing evidence shows that FOXOs are involved in both the autophagy–lysosomal and in the ubiquitin–proteasome pathways.[136–139]

In myotubes, FOXO3A stimulates lysosomal proteolysis by inducing the expression of many autophagy-related genes involved in various steps of the process, including microtubule-associated protein 1 light chain 3 beta (LC3B), GABARAPL1 (GEC1), Beclin 1 (BECN1), phosphatidylinositol 3-kinase 3 (VPS34), unc-51 like autophagy activating kinase 2 (ULK2), and the ubiquitin-like protein ATG12 (APG12L).[140] In mouse skeletal muscle, stimulation of the lysosomal proteolytic pathway leads to atrophy[141] and FOXO3A contributes to this process by activating the Akt1 pathway up-regulating the expression of Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), a Bcl-2 related protein that are involved in the modulation of autophagy in multiple cell systems.[142–145] FOXO3A has been involved in mitophagy as well. In muscles, FOXO3A activates the expression of MUL1, a mitochondrial E3 ubiquitin ligase promoting fragmentation, depolarization, and clearance of mitochondria through the autophagy-lysosome pathway targeting the MFN2 protein in skeletal muscle *in vivo* and in C2C12 myotubes.[146] In the previous section, we have already described the FOXO3A-dependent activation of the PINK1-Parkin pathway in rat cardiomyocites, with consequent increase in the mitochondria fission process and the selective degradation of dysfunctional mitochondria.[135] Additionally, Tseng and colleagues showed that SIRT3-related deacetylation of FOXO3A regulates the expression of the primary mitophagy mediators BNIP3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L or NIX) and LC3 in endothelial cells.[132]

In addition to autophagy and mitophagy, FOXO3A is also involved in the ubiquitin–proteasome system (UPS), a protein quality-control process responsible for the degradation of cytosolic proteins[147] and the removal of damaged proteins and protein aggregates.[148] In skeletal muscle atrophy, FOXO3A directly regulates the expression of the muscle specific E3 ubiquitin ligase MURF1 upon activation mediated by AMPK-α2.[149] FOXO3A can also mediate the crosstalk signalling between the intracellular kinases PI3K/AKT and mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (Erk) pathways to coordinate the transcription levels of ATROGIN-1 (an E3 ubiquitin ligase also known as FBOX32) and Ubiquitin C in glucocorticoid-induced skeletal muscle atrophy.[133]

12.3.3 FOXO3A and Parkinson's Disease

Overall, these data indicate that FOXO3A is at the crossroad of pathways and mechanisms of great relevance for PD etiopathogenesis, including mitochondrial function, redox homeostasis, PINK1 and Parkin biology, protein quality control, and Erk signalling, to mention a few.[80,82,94,150,151] On these premises, the paucity of studies to better evaluate a potential role of FOXO3A in PD is rather surprising; at the time this chapter was written, a PubMed search using the keywords 'FOXO3A' and 'Parkinson' retrieved only six items. Recent studies, however, show that activation of the FOXO3A pathway may have neuroprotective effects in PD signalling models *in vitro* and *in vivo*[152] and, accordingly, that SIRT2 mediated FOXO3A deacetylation, and subsequent suppression of its activity, exacerbates MPTP toxicity in mice.[153] These experimental data substantiate the hypothesis that FOXO3A might participate in PD pathogenesis and pave the way for future and more detailed investigations.

12.4 Role of Other Genes Emerged from Animal Studies in Aging

As in human studies, exploring the genetics of aging in laboratory animal models presents its peculiar challenges. An investigator should always question whether the aging phenotype elicited by a mutation in a single gene is representative of natural aging, or if it rather reflects general deterioration of the organism.[154,155] Additionally, the extent of correlation of a mutation in a single gene with longevity should be carefully pondered.[154,155] Bearing in mind these caveats, studies performed in different laboratory model organisms suggested that several candidate genes might play a relevant role in increasing lifespan and in promoting healthy aging.

The identified genetic mutants have been classified into three different clusters based on their effects on life extension: (1) long-lived mutants that can extend longevity; (2) short-term mutants that are prone to develop aging-related disorders and significantly reduce lifespan, and (3) mutants that have no direct effect on longevity but can be used to study in detail molecular mechanisms and relevant pathways involved in aging processes.[154]

Among them, it is worth highlighting the very well described insulin/IGF-signalling (IIS) system, which has been identified for the first time in *Caenorhabditis elegans*. Mutations in the ISS genes Daf-2 and age-1, and in the FOXO homolog Daf-16 are associated with extensions of lifespan, increased resistance to oxidative stress and reduced oxidative damage.[156–159] Similarly, in *Drosophila melanogaster* and in the long-lived Ames and Snell Dwarf mouse strains, depressed insulin signalling results in increased life extension.[160,161] Accordingly, over-expression of the *Klotho* gene to repress intracellular signalling between insulin and insulin-like growth factor receptor 1 (IGF-1R) in mice leads to lifespan extension.[162] Complete ablation of IGF-1R as well as of the insulin receptor (IR), is highly deleterious because it leads to insulin

resistance and insulin-related disorders such as obesity and diabetes, which are all aging-related conditions that adversely impact lifespan in humans and animal models.[163] Controversially, selective IGF-1R ablation and impairment of the IIS pathway in the CNS decrease amyloid-beta accumulation and prevent mortality in an AD mouse model.[164] In humans, down-regulation of the IIS has been observed in postmortem brain of patient of sporadic AD;[165] however, the identification in centenarians of reduced circulating IGF-1 levels in association to normal insulin production is beneficial for survival in old age,[166–168] suggesting that modulation of the IIS system could reduce the occurrence of age-related diseases, therefore improving the quality of natural aging and extend lifespan.

Biological deterioration typical of aging has also been ascribed to accumulation of macromolecular damage in biological molecules caused by byproducts of metabolism such as ROS. This theory was originally proposed by Denham Harman in 1956, is commonly known as the free-radical theory of aging, and has been held as a plausible model since its original conception.[169] Accumulation in time of ROS-mediated chemical modifications to DNA (*i.e.* DNA damage) could be particularly harmful because it may corrupt the genetic information and therefore the cell function at its core.[170] Consistently, mutations in certain biological pathways responsible for repairing DNA lesions are associated with a phenotype that closely resembles aging, even though it progresses at a much faster pace and is consistently described as accelerated aging. In humans, these defects result in devastating progeroid conditions such as the Cockayne syndrome. Consensus on the extent of affinity between natural and accelerated aging, however, is not unanimous and the topic is still the object of a passionate debate between scholars.[171] The nucleotide excision repair (NER) pathway is a versatile mechanism that is able to amend several types of DNA lesions and may contribute to the pathogenesis of neurodegenerative diseases.[172,173] Mutations in genes involved in the NER system, and in transcription coupled nucleotide excision repair (TC-NER) branch in particular, are often associated with the occurrence of age-related phenotype and aging-related disorders early in life and to a remarkable reduction in lifespan, suggesting that mutations in genes involved in the DNA-repair pathways could play a relevant role in accelerating the aging processes.[174] Disorders such as Cockayne syndrome, xeroderma pigmentosus (XP), or trichothiodystrophy (TTD) are characterized by the progressive appearance of premature aging phenotypes in the first decade of life.[175] In particular, CS, XP and TTD patients develop defects typically associated with aging such as hearing loss, cataract, cachexia, oxidative damage and progressive neurological degeneration in early life and exhibit a severely reduced lifespan.[172,174,176] Several organism models have been developed to dissect in detail the correlation between defects in the NER system, the manifestation of multiple age-related defects, and lifespan.[177–182] These models have also been used to study aging-related disorders,[183–185] despite the fact that the rapidly developing phenotype is in contrast with the chronic, slow progressing one observed in natural aging and in neurodegenerative conditions such as PD.[155]

A further set of evidences connecting DNA damage and aging stems from evidence obtained in a mouse model harboring a defective form of the mitochondrial DNA (mtDNA) polymerase (polymerase gamma or POLG). This enzyme has a 3′-5′ exonuclease activity that is essential to proofread mtDNA and that was ablated in mutant mice. Consequently, this strain is characterized by pronounced accumulation of mutations in mtDNA, was accordingly named the '*mtDNA-mutator*' mouse, and displays premature aging and reduced lifespan.[186,187] This model is interesting also because it establishes a further link between the aging, DNA repair, and the free-radical theory of aging. An implication of this theory is in fact that ROS production, which occurs especially during mitochondrial respiration, may induce mutations in mtDNA, which in turn may impair mitochondrial activity to set off a vicious circle that ultimately leads to further increase in ROS production and thus faster progression of aging.[169] This theory indeed fits with findings showing that specimens from aged individuals often exhibit fewer and structurally abnormal mitochondria,[188] increased oxidative damage,[189] and declined mitochondrial respiration activity.[190,191] In line with these evidences there are observations that long-lived animals show reduced oxidative damage and increased resistance to oxidative stress.[192,193] However, only a minor increase of oxidative damage and normal ROS production levels were observed in the '*mtDNA mutator*' POL-G mutants, weakening the nexus between mt-DNA oxidative damage and premature aging.[187] Similarly, mice deficient in oxoguanine DNA glycosylase (OGG1), a gene responsible for the oxidative DNA lesion 8-hydroxy-2-deoxyguanosine (8-OHdG) removal, show increased mt-DNA oxidation levels compared to wild-type mice, but no signs of increased oxidative stress and regular mitochondrial activity.[194]

Exposure to sources of oxidative stress can certainly lead to aging; a classical example comes from the skin, where UV-exposure constitutes a major contributor to the aging process.[195,196] However, the free-radical theory of aging has been challenged by some recent studies in genetically modified mouse models. In fact, genetic manipulation of antioxidant enzymes failed to conclusively demonstrate direct connections between overexpression of antioxidant genes and longevity as well as between increased level of oxidative stress and reduction in lifespan.[197,198] Null mutants for glutathione peroxidases 1 (GPx1) and MnSOD genes, or heterozygous mutants for glutathione peroxidase 4 (GPx4), for instance, showed no difference in lifespan compared to wild-type animals, despite increased levels of oxidative damage and elevated sensitivity to oxidative stress.[199] Deletion of the CuZnSOD gene showed a 30% reduction of life extension, which was, however, attributable to an increased incidence of hepatocellular carcinoma rather than decreased antioxidant capabilities.[200] Mutation in the thioredoxin 2 gene (Trx2) was associated with a diminished mitochondrial activity, with an increased production in ROS, and with a slight decrease in lifespan (7%).[198,201] Overexpression of MnSOD, CuZnSOD, GPx4, and catalase increased resistance to oxidative stress, yet led to no improvements in longevity.[198] Catalase is normally expressed in peroxisomes and mediates dismutation of H_2O_2 to O_2 and H_2O.

Interestingly, redirection of catalase expression *via* a targeting presequence to the mitochondria (MCAT mice), which normally do not contain this enzyme, resulted in significant delay of age-related anomalies such as cardiac dysfunction and cataract, as well as significant lifespan extension.[202] Subsequent studies further substantiated the original findings.[203–205] Overall, the results in MCAT mice reinforce the concepts that excessive oxidation might indeed favor the aging process and reveal that targeting antioxidant intervention to critical subcellular compartments is imperative for success. Nonetheless, factors other than abatement of oxidative stress might contribute to MCAT mice longevity, as also suggested in the original publication by the authors[202] who speculated that chronic reduction of H_2O_2-mediated intracellular signalling might also participate to the phenotype. Altogether, these findings indicate that further studies are required to unravel the mechanisms integrating redox tolerance, DNA repair, and aging.

12.4.1 Are Aging-Modifying Genes Discovered in Laboratory Animals Relevant for PD?

All the processes mentioned in the previous section have been implicated in PD pathogenesis, at least to some extent, and might therefore be relevant to explain the relationship between aging and this disorder.

The involvement of both exogenous and endogenous oxidative stress in PD etiopathology has been extensively documented.[206–208] Nonetheless, we are still unable to manipulate the cellular redox environment, as evidenced by the disappointing outcome of numerous clinical trials based on antioxidant compounds.[209,210] Failure to operate effective redox control likely reflects the complexity of the network controlling the redox environment, which cannot be maintained at its homeostasis by simply providing electron donors (*i.e.* antioxidant molecules). Manipulation should probably be attempted on different levels, for instance targeting also the signalling pathways that participate in the redox control in a given cell type (*e.g.* DAergic neurons). Future studies will be necessary to address these issues and understand the role of age-related redox alteration on PD pathogenesis.

A role for DNA damage and repair in PD is gradually emerging. Leucine-rich repeat kinase 2 (LRRK2) mutations, for instance, have been found to cause mtDNA-damage. The latter also specifically increases in DAergic neurons of PD patients and PD animal models.[211,212] DNA quality control is an issue of particular relevance for neurons, which are post-mitotic cells and must preserve their DNA lifelong. It is tempting to speculate that DAergic neurons, which are intrinsically more oxidized than other neuronal population even in normal conditions,[80,213] might suffer from a progressively higher burden of DNA-damage accumulation and, in time, might become sensitive to environmental hazards related to PD. Some studies to address this possibility have been already performed,[214] but additional experiments are certainly required to produce conclusive evidence.

Also, the growth hormone and the insulin pathways have been studied in PD. Although no alterations in the growth hormone (GH)/IGF-1 axis have been reported in PD patients, IGF-1 provides protection against DAergic degeneration in PD cellular and rodent models.[215–217] GH production, however, is stimulated by several drugs currently used to mitigate PD symptoms, such as levodopa.[218,219] A potential involvement of GH in the principal levodopa undesired side effect, dyskinesia, has not been explored thus far. Finally, a recent review summarizes the findings suggesting that several pathways deregulated in PD are also altered in diabetes and therefore establishes a mechanistic nexus between these diseases.[220]

The evidence and concepts discussed in this chapter suggest that multiple factors involved in natural aging might constitute potential modifiers of PD pathogenesis. Surprisingly, studies to explore this possibility are relatively uncommon, therefore leaving ample room for future investigations.

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