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The Hsp70 Chaperone System in Parkinson's Disease

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1. Introduction

Several neurodegenerative diseases are associated with a build up of misfolded or abnormal proteins and the formation of distinct aggregates, resulting in a putative pathological protein load on the nervous system (Chiti & Dobson, 2006). This aberrant accumulation of amyloid or amyloid-like aggregates occurs in Parkinson's (PD), Alzheimer's (AD), and Huntington's (HD) diseases, amyotrophic lateral sclerosis, and frontotemporal dementia, among others. A broad array of cellular defence mechanisms operate to counteract this effect, including antioxidant proteins, the stress-inducible response and, in particular, molecular chaperones (Morimoto, 2008; Voisine et al., 2010). Molecular chaperones are responsible for maintaining normal protein homeostasis within the cell by assisting protein folding, inhibiting protein aggregation, and modulating protein degradation pathways (Hartl & Hayer-Hartl, 2009). Currently, there is substantial evidence supporting the involvement of these protein aggregational processes and a role of molecular chaperones, and especially of Hsp70, in PD pathogenesis (Bandopadhyay & de Belleruche, 2010; Broadley & Hartl, 2009; Witt, 2009). Firstly, extensive colocalization of Hsp70 with α -synuclein (α Syn), the major component of Lewy bodies (LBs) (Spillantini et al., 1998), within the intraneuronal inclusions in PD brains has been demonstrated (Auluck et al., 2002; McLean et al., 2002). Secondly, patients with PD show highly perturbed expression of different members of the Hsp70 family in the *substantia nigra pars compacta* (SN) of the brain, which is precisely the target of neurodegeneration (Grunblatt et al., 2001; Hauser et al., 2005). Finally, there is a considerable amount of data derived from studies performed *in vitro*, in cell culture and with animal models of PD (Arawaka et al., 2010; Witt, 2009), that support the protective effects of Hsp70 against α Syn aggregation and toxicity, considered to be central in the aetiology of the disease.

The discovery within the last few years of three different missense mutations (A30P, E46K and A53T) in the α Syn gene as causative of early onset PD unambiguously linked this protein to disease onset and progression (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Additionally, a locus triplication causing an increased dosage of the wild-type (Wt) α Syn gene has been found to potentiate neurodegeneration (Singleton et al.,

2003). Finally, as mentioned above, α Syn is the major component of intracellular protein-rich aggregates found in the brain of post-mortem patients of PD, the LBs and Lewy neurites (LNs). The appealing hypothesis for LBs formation is that α Syn monomers combine to form oligomers (or protofibrils), which coalesce into fibrils and then co-aggregate with other proteins into (intracellular) inclusions (Conway et al., 1998; Wood et al., 1999). While the monomers and oligomers of α Syn are soluble, the fibrils and LBs are insoluble in the neuronal cytoplasm. Some controversy arises, however, from the roles of the various physical forms or species of α Syn in PD pathogenesis. LBs have been proposed to be both neurotoxic (El-Agnaf et al., 1998), and protective (Mouradian, 2002; Rochet et al., 2000). Other hypotheses state that the pre-fibrillar intermediates, composed of α Syn oligomers, are the main toxic species towards dopaminergic neurons (Conway et al., 2000; Volles & Lansbury, 2003). Lansbury and co-workers have shown that α Syn oligomers can form annular, elliptical, or circular amyloid pores in cell membranes (Lashuel et al., 2002a; Volles & Lansbury, 2003), and cell culture studies have demonstrated that α Syn oligomers reduce cell viability, disrupt lysosomes and induce Golgi fragmentation (Gosavi et al., 2002), as well as toxicity in animal models (Karpinar et al., 2009). In line with these findings, the more neurotoxic A30P and A53T mutants of α Syn share an increased tendency to form soluble oligomeric intermediates, whereas the E46K and A53T mutants fibrillate faster than the wild-type protein (Conway et al., 2000; Choi et al., 2004).

The heat-shock-protein 70 (Hsp70) family of chaperones (Mayer & Bukau, 2005; Young, 2010) is well conserved from bacteria to higher eukaryotes (where it is found within different organelles), having critical roles in a range of cellular processes such as promoting the folding of newly synthesized proteins and assisting the rescue of misfolded aggregated proteins. Hsp70 is highly relevant in the context of protein conformational diseases given that stress-induced cytosolic Hsp70 can prevent protein aggregation and enables the cell to avoid the accumulation of potentially toxic aggregates (Hartl, 1996).

The structures of several Hsp70 homologues are similar and consist of an actin-like ATPase domain (nucleotide-binding domain, NBD) and a C-terminal substrate-binding domain (SBD), which are connected by a short linker region (Mayer & Bukau, 2005). The substrate binding pocket recognizes and binds to unstructured or partially folded stretches within polypeptides (Bukau & Horwich, 1998), with the current view that Hsp70s could prevent misfolding by binding to certain patterns in the polypeptide chain of the substrate that are highly enriched in hydrophobic residues (Maeda et al., 2007; Rudiger et al., 1997). Even though most of our current understanding of the Hsp70 molecular mechanism has largely derived from studies performed with the bacterial orthologue (DnaK), the outlines of the mechanism appear conserved (Hartl & Hayer-Hartl, 2002; Young, 2010). The ATPase cycle of Hsp70 involves alternation between an ATP-bound state which has low affinity and fast exchange rates for peptide substrates ('open' state), and an ADP-bound state with high affinity and low exchange rates for substrates ('closed' state) (Mayer & Bukau, 2005). This alternation is achieved by a bidirectional structural communication between the NBD and the SBD domains, driven by a complex allosteric mechanism (Mayer & Bukau, 2005; Young, 2010).

The ATPase cycle is typically modulated by several co-chaperones, most notably the 'J-domain' protein Hsp40/DNAJB1 (or DnaJ, the bacterial orthologue), resulting in increase of the ATPase activity (Bukau & Horwich, 1998; Mayer & Bukau, 2005; Minami et al., 1996), and the BAG family of proteins which function as nucleotide-exchange factors (NEFs) and promote the ADP release from the Hsp70 NBD (Takayama et al., 1999; Young, 2010). In

addition to acting as enhancer of the basal ATPase rate of Hsp70, the Hsp40 family of co-chaperones has a key role in the canonical model of the Hsp70 machinery mechanism, given the ability of some of them to also recognize and bind to unfolded client proteins and 'deliver' them to Hsp70 (Kampinga & Craig, 2010). Other important co-chaperones are Hop, which binds to the C-termini of Hsp70 and Hsp90 and assists substrate transfer between the two chaperones (Scheufler et al., 2000), and Hip (ST13) which has been shown to specifically bind to and stabilize, the ADP-bound state of Hsp70 (Hohfeld et al., 1995; Prapapanich et al., 1996), and has been suggested to increase the half-life of Hsp70-substrate complexes (Hohfeld et al., 1995). Finally, CHIP (Ballinger et al., 1999) acts as an E3-ubiquitin ligase that ubiquitinates HSPA8/Hsc70/Hsp73 (i.e. the constitutive cytosolic Hsp70) substrates, promoting their degradation by the proteasome (Hohfeld et al., 2001).

2. Links between Hsp70 and the pathogenesis of PD

PD is currently thought to involve different pathogenic mechanisms that eventually lead to neurodegeneration, as discussed elsewhere in this book series.. There is substantial evidence supporting a prominent role in PD-related cell death of α Syn toxic oligomers. One hypothesis postulates that certain α Syn species can affect the homeostasis of cell membranes (Gupta et al., 2008; Lashuel et al., 2002b; Volles & Lansbury, 2002), and produce ER and oxidative stress, UPS and mitochondrial dysfunction (Gupta et al., 2008; Jellinger, 2010), as well as neuroinflammation (Jellinger, 2010; Roodveldt et al., 2008) processes, all of which have been linked, to a lower or larger extent, to the amyloid-like aggregation of α Syn. As will be described below, the Hsp70 system has been found to be a key player in counteracting most of these processes, not only by physically interacting with α Syn, but also by promoting aggregation clearance (Figure 1).

Fig. 1. Links between Hsp70 and the multiple α Syn-mediated processes in PD pathogenesis. CMA: chaperone-mediated autophagy; UPS: ubiquitin-proteasome system; LB: Lewy bodies; LN: Lewy neurites. Discontinuous lines depict the possible 'sequestration' of certain chaperones by α Syn aggregating species.

2.1 Hsp70 in modulation of α Syn aggregation and cytotoxicity

Heat-shock proteins (HSPs) prevent and reverse the misfolding and aggregation of proteins, and the Hsp70 family in particular is known to play important roles in protecting neurons from protein aggregation-derived stress (Lu et al., 2010). Therefore, it might not seem surprising that Hsp70 has been found to be linked to several neurodegenerative processes and 'conformational' disorders, including PD (Witt, 2009). Notably, Hsp70 has been shown to colocalize with aggregated α Syn within LBs in brains from PD patients (Auluck et al., 2002), strongly suggesting a role for this chaperone in managing α Syn aggregates in the context of PD (Figure 1). It is then that a substantial portion of the research in the field has focused on the effects of this major cytosolic chaperone on α Syn aggregation and cytotoxicity. Following the discovery that Hsp70 can abrogate the neurotoxicity of abnormal polyglutamine proteins (Warrick et al., 1999), it was shown that Hsp70 can also prevent dopaminergic neuronal loss associated with α Syn in a *Drosophila* PD model (Auluck et al., 2002). Numerous studies that followed have reported that over-expression of Hsp70 is able to reduce α Syn aggregation and/or toxicity in various cellular models (Danzer et al., 2011; Klucken et al., 2004b; McLean et al., 2004; Opazo et al., 2008; Outeiro et al., 2008; Zhou et al., 2004). In particular, McLean and co-workers (Outeiro et al., 2008) have found that Hsp70 rescues α Syn-linked toxicity by promoting the cellular clearance of α Syn oligomers, rather than monomers. Another study (Opazo et al., 2008) found that Hsp70 manages α Syn intracellular aggregation by increasing the clearing of aggregates primarily through the aggresome, and the subsequent removal of small aggregates and aggresomes from the cytosol. An interesting study by McLean and colleagues (Danzer et al., 2011) has recently shown that Hsp70 can also inhibit the formation of extracellular α Syn oligomers and rescue the cytotoxicity produced by such secreted oligomers, in a cellular model. Moreover, their data also indicates that Hsp70 is released to the extracellular medium together with secreted α Syn, adding to the accumulating evidence that Hsp70 can be released from cells by an active mechanism, with functionally relevant consequences.

Intriguingly, while it was found that over-expression of Hsp70 can prevent α Syn aggregation in a Wt α Syn transgenic mouse model (Klucken et al., 2004b), a recent work based on A53T- α Syn transgenic mice has failed to observe this effect (Shimshek et al., 2010), seeding some controversy. Could these findings reflect a difference in the nature of the aggregates generated by Wt and mutant α Syn, and therefore a differential ability of Hsp70 to cope with those aggregates? Even though there is compelling evidence demonstrating the important role of Hsp70 under physiological and pathological scenarios in modulating fibril formation based on *in vivo* and cellular models, the molecular mechanism underlying such anti-aggregation properties in the context of PD, is still not fully understood.

Unlike the many research works performed with PD cellular and animal models that have contributed to our understanding on Hsp70 function under physiological or pathological conditions, only a handful of studies have focused on the molecular mechanism and interactions that underlie the modulation of α Syn aggregation exerted by Hsp70. An in-cell fluorescence resonance energy transfer (FRET) study indicated that Hsp70 alters the conformation of α Syn, inducing it to adopt a more 'open' conformation, without affecting the α Syn- α Syn intermolecular interactions (Klucken et al., 2006). Similarly to what had been found *in vitro* for Hsp70 and the HD-related huntingtin protein (Muchowski et al., 2000; Wacker et al., 2004), several studies with α Syn have shown that Hsp70 is able to suppress α Syn fibril assembly. In this case, a variety of *in vitro* studies have shown that this efficient

inhibition of amyloid assembly can occur in the absence of collaborating co-chaperones and in an ATP-independent manner (Ahmad, 2010; Dedmon et al., 2005; Huang et al., 2006; Luk et al., 2008; Roodveldt et al., 2009), while promoting formation of small oligomeric species of moderate toxicity (Roodveldt et al., 2009). However, our recent study demonstrates that, even though Hsp70 is indeed able to control α Syn fibrillation and its associated toxicity in an ATP-independent manner, it does so more efficiently in the presence of ATP (Roodveldt et al., 2009), consistent with the results obtained with a cellular model (Klucken et al., 2004a). Nevertheless, this ATP-dependent activity was found to require Hip co-chaperone activity, and not Hsp40 as observed in the case of mutant huntingtin protein (Lotz et al., 2010), suggesting that Hsp70 can function through different molecular mechanisms, depending on the nature of the aggregating client protein.

Interestingly, our study also showed that Hsp70 in the presence of ATP is prone to co-aggregate with aggregating α Syn, presumably caused by the formation of a highly insoluble (ADP)Hsp70/ α Syn complex. Surprisingly, this co-aggregation can be prevented by the addition of the co-chaperone Hip (St13), which had been found to be under-expressed in serum of PD patients since the early stages of the disease (Scherzer et al., 2007). The relevance of Hip in assisting the suppression of α Syn aggregation in an Hsp70-dependent manner was further supported by a study we performed in a *C. elegans* model of PD (Roodveldt et al., 2009), in which knock-down of Hip produced a much stronger PD phenotype than knock-down of Hsp70, shown by higher levels of α Syn aggregation in the former condition. This finding indicates that Hip co-chaperone could be important not only in modulating the chaperone's molecular mechanism, but also in guarding the functionality and availability of Hsp70 under certain conditions.

One relevant question relates to the nature of the species along the aggregation pathway of α Syn that are specifically recognized and targeted by Hsp70. Initially, Hsp70 was reported to bind to α Syn filaments *in vitro* (Lindersson et al., 2004). However, it is currently understood that Hsp70 does not disaggregate or alter the structural properties of mature α Syn fibrils (Dedmon et al., 2005), but rather inhibits fibril formation via interactions with soluble pre-fibrillar forms of α Syn. A few studies performed with cell extracts (Zhou et al., 2004) and with live cells (Klucken et al., 2006), in addition to experiments with purified proteins (Dedmon et al., 2005; Huang et al., 2006; Roodveldt et al., 2009), demonstrate the existence of molecular interactions between Hsp70 and α Syn oligomeric species. Even though previous attempts to isolate such complexes by co-immunoprecipitation or pull-down experiments had failed (Luk et al., 2008), we have been able to probe the formation of a complex between Hsp70 and α Syn oligomeric species by FRET (Roodveldt et al., 2009), suggesting that these interactions are transient (Luk et al., 2008; Roodveldt et al., 2009), besides being highly dynamic (Luk et al., 2008; Roodveldt et al., 2009).

In addition, we have recently shown by FRET and nuclear magnetic resonance (NMR) analyses that, contrary to what was previously thought, Hsp70 can also interact with α Syn monomeric species (Roodveldt et al., 2009) with an affinity constant lying within the low micromolar range. Moreover, the fluorimetric study revealed the existence of diverse Hsp70/ α Syn complexes that are formed depending on the nucleotide state of the chaperone, either nucleotide-free, or ATP- or ADP-bound, that are sampled along the ATPase cycle. Based on these structural studies, we have proposed that a particularly 'compact' Hsp70/ α Syn complex in the ADP-state of Hsp70 that arises during the aggregation process of α Syn, leads to Hsp70 being entrapped or sequestered by the oligomers. However, we

propose that this compact complex can be stabilized by the Hip co-chaperone and therefore the co-aggregation of the chaperone, and ultimately the formation of α Syn fibrils, can be prevented (Roodveldt et al., 2009). In other words, our findings indicated that a decreased expression of Hip could facilitate depletion of Hsp70 by amyloidogenic polypeptides, impairing chaperone proteostasis. Interestingly, another case of chaperone-inhibition/depletion by α Syn oligomers, but not by unstructured monomers, was recently reported for the Hsp70/Hsp40 system (Hinault et al., 2010). In this case, the authors found that the inhibition of the chaperone system was predominantly caused by the sequestration or incapacitation, by off-pathway α Syn oligomers, of the J-domain (Hsp40) co-chaperone (Hinault et al., 2010).

What is the region on the α Syn molecule thought to be recognized and bound by Hsp70? One study mapped this region as the broad segment between residues 21 and 110 (Luk et al., 2008). Based on a predictive algorithm for Hsp70-binding regions (Rudiger et al., 1997) and FRET analyses to probe Hsp70- α Syn interactions (Roodveldt et al., 2009), our results indicate that Hsp70 can bind to the N-terminus and the central NAC region of the protein. The first binding region is involved in functional lipid interactions, while the second one comprises the stretch of hydrophobic residues that readily forms fibrils *in vitro* and is generally assumed to be involved in initiating the fibrillation process (Giasson et al., 2001). In addition, our results suggest that Hsp70 also interacts with the negatively charged C-terminus of the α Syn molecule, especially in the nucleotide-free state. Taken together, the studies carried out thus far demonstrate that Hsp70 modulates α Syn aggregation by interacting with the protein at different stages of aggregation, by recognizing essentially two or three regions in the α Syn molecule, and forming different transient complexes with the substrate. The strong binding versatility displayed by Hsp70 with aggregating α Syn might be possible thanks to the large structural flexibility conferred by the lid subdomain within the SBD, that has been recently described by Mayer and colleagues (Schlecht et al., 2011).

2.2 Cooperation of Hsp70 with the ubiquitin-proteasome system (UPS)

2.2.1 Involvement of the ubiquitin-proteasome system in PD

The ubiquitin-proteasome system (UPS) is responsible for the degradation of vital regulatory proteins that control almost every cellular function (Hershko & Ciechanover, 1998). The UPS system is composed of three classes of ubiquitinating enzymes (E1, E2 and E3) that activate, transfer and attach the small protein ubiquitin to the Lys residues of proteins that are targeted for degradation. Conjugation of at least four ubiquitin moieties acts as the degradation signal in a process that is initiated by the recognition of the ubiquitin linkage by the proteasome, which first catalyzes the unfolding and then the proteolysis of the targeted protein (Hershko & Ciechanover, 1998).

Impairment in the UPS has been linked to many neurodegenerative disorders and indeed to contribute to disease progression in PD (Ciechanover & Brundin, 2003; Cook & Petrucelli, 2009; McNaught et al., 2001). The involvement of UPS in PD arouse major interest with the identification of mutations in the E3 ubiquitin ligase parkin protein as a cause of autosomal recessive PD (Kitada et al., 1998). Both the loss of E3 activity and the possibility of incomplete or aberrant ubiquitination are proposed as causes of parkin-related PD (Giasson & Lee, 2003). A second member of the UPS involved in PD is the ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1), and mutations in the *uchl-1* gene cause dysfunction of this enzyme and lead to accumulation of toxic products (Leroy et al., 1998).

Beyond the clear genetic association between the UPS and PD, several studies have demonstrated a reduced proteolytic activity in the SN of PD patients when compared with aged-matched controls (McNaught et al., 2003; McNaught & Jenner, 2001; McNaught & Olanow, 2006). In culture of rat primary neurons, treatment with proteasomal inhibitors has shown to lead to the formation of α Syn- and ubiquitin- positive proteinaceous inclusions, analogous to LBs found in PD patients (McNaught et al., 2002a; Rideout et al., 2005). Moreover, systemic administration of proteasomal inhibitors in rats produced a behavioural and pathological phenotype strongly reminiscent of PD (McNaught et al., 2004), although recently some controversy arose in this matter (Manning-Bog et al., 2006). Further support to the active role played by proteasomal impairment in PD progression originated from studies with pesticides, such as rotenone and paraquat (Cook & Petrucelli, 2009). Mice treated with such environmental toxins display a strong reduction in proteolytic activity that is dependent on the presence of α Syn (Fornai et al., 2005). All together, these data undoubtedly link α Syn aggregation with impairment of the UPS in PD progression.

In relation to protein misfolding and aggregation, it has been proposed that failure of the UPS to adequately remove misfolded or abnormal proteins may underlie demise of nigral cells in sporadic PD (McNaught et al., 2001). Furthermore, deficits in the 26/20S proteasome pathways are accompanied by protein accumulation and aggregation, which may also cause neurodegeneration (Chung et al., 2001), in line with recent findings that general intracellular aggregation of proteins into aggresomes can inhibit the UPS (Bence et al., 2001). Moreover, chaperones of the heat-shock families, including HSP27, 40, 70, 60, 90, and 110, as well as components of the UPS, such as ubiquitin, UCH-L1 and parkin, are found in LBs extracted from PD patients *post mortem* (Shults, 2006). These combined pieces of evidence have attracted much attention lately as they imply that LBs could originate from ubiquitin-rich aggresomes that the proteasomal components may not be able to process (McNaught et al., 2002b). One plausible mechanism would involve α Syn adopting abnormal protein conformations and overwhelming the cellular protein degradation systems (Wong & Cuervo, 2010), whereas deficits in the UPS machinery would challenge the cell's ability to detect and degrade misfolded proteins that can result in the formation of toxic early aggregates (McNaught et al., 2002b). The common outcome of this failure at different levels is thus expected to be a cellular build-up of unwanted toxic species that should have been cleared in otherwise healthy conditions. Minimal defects in the crucial protein turnover machinery may suffice to cause a slow demise of dopaminergic neurons, which may explain the relentless, progressive nature of the disease (Vila & Przedborski, 2003).

Alpha-synuclein extracted from LBs in PD brains has been found to be mono- and di-ubiquitinated (Hasegawa et al., 2002), while soluble α Syn is mono-ubiquitinated by SIAH-1 and -2, but not by parkin (Liani et al., 2004; Rott et al., 2008). Instead, a modified, O-glycosylated, version of α Syn is a substrate for parkin-induced ubiquitination (Shimura et al., 2001), and it was shown that interaction of parkin with α Syn is mediated by synphilin-1 (Chung et al., 2001) as well as by the protein 14-3-3- η (Sato et al., 2006). Importantly, parkin has been shown to be able to rescue primary neurons from the toxic effects of α Syn (Petrucelli et al., 2002), suggesting that the two proteins share a common pathway that may determine the fate of dopaminergic neurons in PD. In addition, UCH-L1 may be involved in regulating the cytoplasmic abundance of α Syn, as it displays unexpected ubiquitin ligase activity that is also able to polyubiquitinate mono- and diubiquitinated α Syn (Liu et al., 2002). Since attachment of at least four ubiquitin molecules is known to be required for

protein degradation via the UPS (Hershko & Ciechanover, 1998), it is likely that mono-, di-, and aberrant poly-ubiquitinated α Syn could have a pathogenic impact (Rott et al., 2008; Sun & Chen, 2004).

2.2.2 Role of the C-terminus Hsp70-interacting protein (CHIP) in PD

Hsp70 and Hsp90 family members as well as small HSPs all take part in the degradation of protein substrate and are able to cooperate with the UPS towards this goal (Patterson & Hohfeld, 2006). Notably, protein homeostasis thus appears to be tightly controlled by interplay between the protein folding and protein degradation systems. Hsp70 takes part in the degradation of immature and aberrant forms of certain proteins, particularly ER-bound membrane proteins (Taxis et al., 2003), but also some cytosolic and nuclear proteins (Bercovich et al., 1997). For example, Hsp70 assists in the folding of the aggregation prone cystic fibrosis transmembrane conductance regulator (CFTR); however, Hsp70 is also able to present CFTR to the UPS and thus to control precisely the abundance of this protein, which is known to accumulate in aggresome and to cause cystic fibrosis (Zhang et al., 2001). Indeed, Hsp70 is actively recruited to aggresome and it has been proposed that this chaperone can reduce aggresome formation by stimulating proteasomal degradation of misfolded proteins (Dul et al., 2001; Garcia-Mata et al., 1999). The proposed mechanism for chaperone-UPS cooperation is that both systems compete for the same misfolded and aggregation-prone substrate proteins, and that the efficiency of chaperones in maintaining these proteins in solution increments the probability of the UPS to degrade aberrant polypeptides. Conversely, failure of chaperones to keep misfolded proteins in a soluble state gives rise to aggregates that are not efficiently degraded by the UPS and, moreover, may inhibit UPS activity towards other protein targets, altering protein homeostasis (Figure 1).

Insights into the mechanism that enables the cooperation of protein chaperones with the UPS have been obtained from the identification and functional characterization of the C-terminal Hsp70 interacting protein (CHIP) co-chaperone (Ballinger et al., 1999; Hohfeld et al., 2001; McDonough & Patterson, 2003). CHIP contains three tetratricopeptide repeat (TPR) domains that recognize and bind to the EEVD motif in both Hsp70 and Hsp90 (Ballinger et al., 1999). CHIP-complexed Hsp70 displays reduced ATP hydrolysis *in vitro*, suggesting that the co-chaperone diminishes the on-rate of binding and release cycles in Hsp70 (Ballinger et al., 1999). In its C-terminus, CHIP possesses a ubiquitin ligase domain (U box) that is capable of targeting proteins to degradation, in a homologous manner to RING finger domains found in E3 ubiquitin ligase enzymes (Connell et al., 2001; Meacham et al., 2001).

CHIP has been shown to efficiently act as E3 ligase for several Hsp70 and Hsp90 substrates, such as the glucocorticoid receptor and Erb2 (Dickey et al., 2007; McDonough & Patterson, 2003; Murata et al., 2001). Proteins ubiquitinated by CHIP are efficiently targeted to the proteasome and subsequently degraded. Notably, the UbcH4/UbcH5 proteins are E2-conjugating enzymes (Demand et al., 2001) that are stress-activated, suggesting that upon stress CHIP furnishes the cell with chaperone-dependent ubiquitin ligases capable of ubiquitinating misfolded and aggregation-prone substrates.

The ubiquitinating activity of CHIP is regulated by two co-chaperones, BAG-1, which is an enhancer, and the Hsp70 binding protein 1 (HspBP1), which acts as a repressor. BAG-1 associates with the ATPase domain of Hsp70 and, in addition, possesses a ubiquitin-like domain that is efficiently recognized and bound by the proteasome (Luders et al., 2000). Thus, BAG-1 provides the proteasome with the capability to recruit Hsp70 and consequently

degrade its cargo clients. A ternary complex involving BAG-1, Hsp70 and CHIP has been isolated, and BAG-1 is capable of stimulating CHIP mediated degradation of some proteins (Alberti et al., 2002). The co-chaperone HspBP1, in turn, competes with BAG-1 for binding to the ATPase domain of Hsp70 (Alberti et al., 2004). Notably, when in complex with HspBP1-bound Hsp70, CHIP shows a much reduced ubiquitin ligase activity and chaperone substrates are not longer ubiquitinated and targeted to the UPS (Alberti et al., 2004).

In the context of neurodegeneration, CHIP has been shown to intervene in the degradation of misfolded aggregation-prone proteins associated with AD, PD and HD, as well as with spinocerebellar ataxia and spinal bulbar muscle atrophy (Adachi et al., 2007; Al-Ramahi et al., 2006; Hatakeyama et al., 2004; Shin et al., 2005; Urushitani et al., 2004). Indeed, CHIP appears to play an active role in modulating α Syn aggregation and degradation (Kalia et al., 2011; Shin et al., 2005; Tetzlaff et al., 2008). It was shown that CHIP interacts with both soluble and aggregated α Syn, and its over-expression reduces α Syn abundance and aggregation (Shin et al., 2005). Interestingly, CHIP induces α Syn clearance via two alternative pathways, one involving Hsp70 and proteasomal degradation, and another Hsp70-independent route that targets α Syn to lysosomes (Shin et al., 2005). A fluorescence-complementation assay demonstrated that CHIP reduced significantly the abundance of α Syn toxic oligomers in cell culture, suggesting that this aberrant species is preferentially recognized by the co-chaperone (Tetzlaff et al., 2008). More recently, it was discovered that CHIP is an E3 ubiquitin ligase of α Syn since it efficiently conjugates ubiquitin moieties to this protein (Kalia et al., 2011). CHIP-catalyzed ubiquitinated forms of α Syn include mono- and poly-ubiquitinated species, and the activity of CHIP depends on the presence of Hsp70 and the co-chaperone BAG5. Contrary to the enhancer activity reported for BAG1, BAG5 reduces the ability of CHIP to ubiquitinate α Syn in an Hsp70-dependent manner (Kalia et al., 2011).

Additional pathogenic mechanisms in PD that involve the activity of CHIP relate to the ubiquitin ligase activity of parkin and the kinase activity of the leucine-rich repeat kinase-2 (LRRK2). It has been found that CHIP, Hsp70 and parkin form a ternary complex that promotes ubiquitination and degradation of the Pael receptor, a protein localized in the ER and whose accumulation has been linked to dopaminergic neuronal death (Imai et al., 2002), and CHIP has been proposed to enhance the activity of parkin, even in the absence of Hsp70 (Imai et al., 2002). Concerning LRRK2, CHIP regulates the ubiquitination, degradation, and toxicity mediated by pathogenic mutations of this kinase (Ko et al., 2009). Moreover, it has been shown that CHIP binds to LRRK2 via its TPR motifs and formation of this complex is protective in cell culture models, while in the presence of mutant LRRK2, knock-down of CHIP leads to cell death (Ko et al., 2009).

2.3 Hsp70 in chaperone-mediated autophagy (CMA)

Macroautophagy and chaperone-mediated autophagy (CMA) are the two main lysosomal proteolytic systems in mammalian cells for the degradation of intracellular proteins (Xilouri & Stefanis, 2011). CMA is the process of degradation of intracellular components by lysosomes which selectively degrades cytosolic proteins containing a KFERQ-like motif (Koga & Cuervo, 2010). This process is known to involve binding of a complex of constitutive cytosolic Hsp70 (Hsc70) and co-chaperones (including Hsp40, Hip, Hsp90 and BAG1) to substrate proteins, and their subsequent targeting to lysosomes via the lysosomal surface receptor LAMP-2A (Xilouri & Stefanis, 2011). The substrate protein is subsequently

degraded after unfolding and translocation into the lysosomal lumen, in a process involving lysosomal Hsc70 (Figure 1). It has been estimated that about 30% of cytosolic proteins could be subjected to degradation via CMA (Dice, 2007), and furthermore, this pathway may be a major route by which α Syn is degraded in neurons (Witt, 2009). Even though it is currently accepted that dysregulation of autophagy plays a role in neurodegeneration (Bandyopadhyay & Cuervo, 2007; Nixon, 2006; Rubinstein, 2006; Xilouri & Stefanis, 2011), including the PD neurodegenerative process (Martinez-Vicente et al., 2008; Yang et al., 2009), the mechanism by which CMA modulates neuronal survival or death, is still unclear. Given that pathologic accumulation of α Syn is a hallmark of PD, several recent studies have addressed the possible link between α Syn degradation, CMA dysfunction and the neurodegenerative process. Indeed, α Syn, which contains a pentapeptide sequence (₉₅VKKDQ₉₉) consistent with Hsc70 binding (Dice, 1990), has been shown to be degraded via CMA using isolated lysosomal preparations (Cuervo et al., 2004) and neuronal cells (Alvarez-Erviti et al., 2010; Martinez-Vicente et al.; Vogiatzi et al., 2008). On the contrary, the A30P and A53T α Syn variants were observed to bind strongly to LAMP-2A receptors but were not internalized, thus inhibiting the CMA degradation of other substrates (Cuervo et al., 2004). This CMA dysfunction was later shown to mediate α Syn toxicity in cellular models (Xilouri et al., 2009). Moreover, a recent study using both α Syn transgenic- and paraquat- PD mouse models (Mak et al., 2010), showed that α Syn can be degraded in the lysosome through CMA, *in vivo*. The study also revealed an up-regulation of LAMP-2A and lysosomal Hsc70 and an increase in Hsc70- α Syn interactions in brain lysosomes, relative to controls. On the other hand, a recent work revealed a significant reduction of both LAMP-2A and Hsc70 levels in the SN and amygdala of PD brains, relative to age-matched Alzheimer's disease (AD) and healthy, brain controls (Alvarez-Erviti et al., 2010). Even though their results might initially appear contradictory, these findings support a key role for Hsc70 and the CMA system in maintaining intracellular general proteostasis, especially within the α Syn-overload scenario that is typically associated to PD and other α -synucleinopathies.

2.4 Emerging links between the Hsp70 system and neurodegeneration in PD

2.4.1 The CSP α -Hsc70-SGT complex and neurodegeneration in PD

Cysteine-string protein α (CSP α) is an abundant protein localized in synaptic vesicles that ameliorates neurodegeneration in cellular and animal models (Johnson et al., 2010). It contains a Dna-J domain and has been shown to interact with Hsc70 and to increase its ATPase activity (Braun et al., 1996). On the other hand, CSP α was shown to assemble into an enzymatically active ternary complex with Hsc70 and SGT (small glutamine-rich tetratricopeptide repeat domain protein) with a likely regulatory function in secretory vesicles (Tobaben et al., 2001). CSP α has also been reported to interact with other chaperones, including Hsp90 (Sakisaka et al., 2002), Hip, Hop (Rosales-Hernandez et al., 2009), and Hsp40 (Gibbs et al., 2009).

CSP α dysfunction has been implicated in various pathologies, including memory impairment, type-2 diabetes, cystic fibrosis, and HD (reviewed in (Johnson et al., 2010)). A few years ago, a link between CSP α and PD was also established (Chandra et al., 2005). The results of this study, performed using a transgenic mouse model, indicate that α Syn cooperates with CSP α in preventing neurodegeneration (Chandra et al., 2005). In a recent study (Sharma et al., 2011), the CSP α -Hsc70-SGT complex was found to bind to monomeric

SNAP-25, a pre-synaptic SNARE protein, and prevent its aggregation, thus promoting the formation of the SNARE complex involved in neuronal synapse. Interestingly, another recent study using a transgenic mouse model of α Syn aggregation with associated neurodegeneration, has shown that SNAP-25 is redistributed within synaptic terminals and the protein was found to colocalize with α Syn within intraneuronal aggregates (Garcia-Reitbock et al., 2010). These data raise the question whether aggregating α Syn might actually be sequestering CSP α involved in the CSP α -Hsc70-SGT complex, analogously to what was observed for aggregating huntingtin (Miller et al., 2003), and therefore enabling the aggregation of SNAP-25 in a PD scenario.

2.4.2 The unfolded protein response (UPR) in PD

The unfolded protein response (UPR) is a mechanism activated within the cell when the endoplasmic reticulum (ER) function is impaired and, as a result, unfolded proteins accumulate in the ER lumen (a process called 'ER stress'). The ER-resident Hsp70 family member GRP78 (HSPA5/BiP) recognizes and binds to such unfolded proteins, which causes the release of UPR-activating factors (Rutkowski & Kaufman, 2004). To restore ER function, the UPR reduces protein translation and enhances the folding and processing capacities within ER. However, if the stress overwhelms the cell restoring capacity, the UPR induces apoptosis (Paschen & Mengesdorf, 2005). A few years ago, a mutation in the parkin gene was found to be related to familial PD due to the impairment of its ubiquitin ligase function which results in protein accumulation within the ER lumen and leads to ER stress and apoptosis (Imai et al., 2001). More recently, a strong correlation between UPR activation and PD pathogenesis in PD patients was established (Hoozemans et al., 2007), suggesting a functional connection between α Syn and ER stress. In addition, it was found that overexpression of (Wt) α Syn triggers UPR in yeast (Cooper et al., 2006). A recent study has shown that α Syn is also found aggregated and accumulated within the ER lumen and induces UPR by binding to GRP78/BiP in cells, which could lead to apoptosis (Bellucci et al., 2011). Clearly, supplementary investigations are needed to determine the exact role of the UPR in the pathophysiology of PD.

2.4.3 Mitochondrial dysfunction in PD

In both familial and sporadic forms of PD, several mitochondrial alterations and increase of oxidative species are well recorded (Jellinger, 2010). The important role of mitochondrial pathology in PD is reflected by the specific and selective loss of mitochondrial complex I activity in the SN of PD patients (Valente et al., 2004). Results from a study with neuronal cell cultures indicate that this impairment is dependent on α Syn mitochondrial import and accumulation (Devi et al., 2008). Interestingly, a recent proteomic study revealed that expression of mortalin (HSPA9/mtHSP70/GRP75), a mitochondrial stress protein and member of the Hsp70 chaperone family which binds to DJ-1 and α Syn (Jin et al., 2007), is significantly decreased in PD brains (De Mena et al., 2009; Jin et al., 2006) as well as in a cellular model of PD (Jin et al., 2006). Moreover, specific coding mutational variants of the mortalin gene have been recently discovered in a few PD patients (Burbulla et al., 2010; De Mena et al., 2009). Finally, differential levels of mitochondrial mortalin were measured in Wt and A53T cellular models of PD (Pennington et al., 2010), suggesting a possible involvement of α Syn aggregation in PD-related mitochondrial dysfunction (Xie et al., 2010).

3. The Hsp70 machinery members as biomarkers of PD

A biomarker is a naturally occurring molecule, gene, or characteristic by which a particular medical condition, disease, etc. can be identified. Despite the current relevance of identifying a biomarker for early diagnosis of PD and/or to follow up its progression, up to date there is no reliable biomarker available (Morgan et al., 2010; Nyhlen et al., 2010). Therefore, certain key proteins that are thought to be tightly linked to PD pathogenesis or progression, such as the members of the Hsp70 machinery discussed above, which could manifest changes in their expression levels in body fluids cells or alter their presence in body fluids in a PD scenario, could represent potential markers of disease development or predisposition.

Currently, it is well established the most promising biomarkers for PD in cerebrospinal fluidic (CSF) are α Syn, DJ-1, amyloid β , and the tau protein. These are the principal targets in the Parkinson's Progression Markers Initiative, a public-private, large-scale study project that aims to identify biological markers of disease progression (www.PPMI-info.org). DJ-1, which is the only chaperone to be included in this study, is a mitochondrial chaperone which has been one of the most studied proteins for its potential use as a PD biomarker. However, results published thus far from measurements of DJ-1 in CSF (Hong et al., 2010) and serum from PD patients (Hong et al., 2010; Shi et al., 2010; Waragai et al., 2007) are somewhat controversial or inconsistent, which could probably be explained by the high DJ-1 protein level present in blood cells (Shi et al., 2010).

Currently, it is well established that certain proteins, including members of the Hsp70 family such as Hsp701A and 1B, display perturbed expression levels in the SN of PD brains (Hauser et al., 2005); however, changes in tissue expression levels are in principle not useful for an application as biomarkers. Recently, a significant decrease in whole blood mRNA levels of St13/Hip co-chaperone was reported for early PD patients, but not for AD patients or healthy controls (Scherzer et al., 2007). However, a second group reported no significant differences in the expression pattern of ST13 in early-stage PD patients, as compared to controls (Shadrina et al., 2010). This discrepancy could be attributed, at least in part, to heterogeneity in the criteria of diagnosing and classifying the individuals into groups and to the difficulty in establishing the actual onset of the disease. Yet another study found differences in HSPA8 (Hsc70) and HIP2 expression levels between PD patients and controls (Grunblatt et al., 2010). Other members of the Hsp70 machinery are known to change their expression patterns in PD patients compared to healthy controls (Hauser et al., 2005). Unfortunately, these changes appear not specific or sufficient to differentiate between PD and other related neurodegenerative disorders (Hauser et al., 2005). This is probably due to the fact that the Hsp70 system plays a central role in maintaining cell proteostasis, which is perturbed in a variety of neurodegenerative diseases.

Considering that PD is a complex pathology that involves several systems such as the stress response, the UPS, the immune system, etc., a unique biomarker might not be enough as a tool for diagnosis or follow-up of disease progression. Instead, there is general consensus that the use of a set of distinct parameters, such as protein expression profiles, age, symptoms and others, would probably be the best approach (Fasano et al., 2008; Grunblatt et al., 2010; Scherzer et al., 2007).

4. The Hsp70 system as a therapeutic tool for PD

As described in this chapter, in the last few years it has become evident that HSPs play an important role in the initiation and progression of PD and other neurodegenerative diseases. This cumulative evidence has prompted the development of therapeutic tools based on the Hsp70 machinery. Different strategies have been tested to manipulate Hsp70 as a therapeutic approximation for PD and related neurodegenerative diseases (reviewed in Kalia 2010). Three general approaches have been explored, namely, to increase the intracellular activity of Hsp70, to overexpress Hsp70 and/or other co-chaperones, and to deliver chaperones or regulatory factors using cell-penetrating peptides (CPPs).

4.1 Increasing the intracellular activity of Hsp70

Theoretically, it should be feasible to control the activity of the cellular chaperone machinery by using different types of drugs. The mechanism of most of such chemical compounds is based on activating HSF-1, a key transcriptional regulator of the heat shock response (HSR) that activates the gene expression of inducible HSPs. One way of activating HSF-1 is by inhibiting Hsp90 activity; as a result, HSF-1 becomes active and increases the expression of inducible chaperones like HSPA1A/Hsp72 and others. Geldanamycin (GA) and its derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) are antibiotics with high affinity for the ATPase domain of Hsp90, which blocks its interaction with HSF-1 and subsequently allows its activation (Waza et al., 2006; Zou et al., 1998). GA prevents α Syn-induced dopaminergic cell loss in cell culture (McLean et al., 2004) and in animal models of PD (Auluck et al., 2005; Shen et al., 2005), while the less toxic 17-AAG has also proven to be neuroprotective in PD cellular models (Danzer et al., 2011; Riedel et al., 2010) and in two animal models of PD-related neurodegenerative diseases (Fujikake et al., 2008; Waza et al., 2005). Even though 17-AAG is currently under phase-II clinical trials as an anti-tumour drug (Pacey et al., 2010; Richardson et al., 2010; Solit et al., 2008), its use in patients with neurodegenerative diseases could be hampered by its toxicity and unavailability for oral administration (Pacey et al., 2010). Another family of inhibitors of Hsp90 activity, is SNX-2112 and its analogues, which are orally available and present improved blood brain barrier (BBB) permeability. In particular, SNX-0723 was shown to prevent α Syn oligomer formation and α Syn-induced toxicity in cell culture (Putchu et al., 2010), and preclinical studies for cancer therapy are proving their safety (Zhai et al., 2011), although similar studies in neurodegenerative animal models are still needed.

Other drugs are able to activate HSF-1 without inhibiting Hsp90 activity, which could represent a less toxic approximation for neurodegenerative pathologies. Arimoclomol, for example, has already been tested in phase I- and IIa-clinical trials for treating ALS, and shown to be safe and tolerable (Cudkowicz et al., 2008; Phukan, 2010). Another example is HSF-1A (Neef et al., 2010), shown to upregulate Hsp70 expression and to reduce poly-Q-citotoxicity in cell and fly models of poly-Q neurodegenerative disorders (Neef et al., 2010). Celastrol, yet a similar drug, and some structural relatives, appear as promising drugs due to their rapid kinetics and low EC₅₀ (Westerheide et al., 2004), although further studies are needed.

Besides these approaches, HSF-1 co-inducers could represent more tolerable drugs for therapy. These are molecules that partially activate HSF-1, reducing its activation threshold and often working in conjunction with secondary stress signals to fully induce HSR. Non steroidal anti-

inflammatory drugs (NSAIDs) are well known co-inducers of HSR. For example, Sodium salicylate and Indomethacin induce HSF-1 DNA binding and reduce the temperature required for triggering the HSR (Jurivich et al., 1995; Lee et al., 1995). The association of NSAIDs use with a lower risk of common neurodegenerative diseases such as AD and PD has been analysed in several studies (Etminan et al., 2008; Gagne & Power, 2010). According to one hypothesis, this negative correlation could be due to continuous up-regulation of HSR and consequently continued cytoprotection against neurodegeneration (Westerheide et al., 2004).

4.2 Overexpression of Hsp70 and/or related co-chaperones

In principle, it should be possible to design a gene therapy approach for the treatment of PD and other conformational neurodegenerative diseases based on HSPs, considering the substantial number of reports having characterized the molecular pathways by which Hsp70 acts in the context of disease. Intriguingly, even though overexpression of Hsp70 has been shown to be protective in animal models of PD (Dong et al., 2005; Jung et al., 2008), a recent study in mouse indicates otherwise (Shimshek et al.). Up to date, one phase-I study in PD patients using recombinant Adeno-Associated Virus (AAV) to deliver aromatic aminoacid decarboxylase enzyme into the putamen, supports the proof-of-principle for the use of gene therapy in PD (Christine et al., 2009). With this precedent, gene therapy could potentially be employed to overexpress other chaperones and co-chaperones that may improve Hsp70 function and its neuroprotective properties. Indeed, recombinant AAV has been already used to transduce Hsp104 (Vashist et al., 2010), a non-mammalian chaperone, in a rat model of PD and proven to be neuroprotective by disaggregating protein inclusions and synergizing with endogenous Hsp70 (Lo Bianco et al., 2008).

Given that co-chaperone BAG-5 is known as a negative regulator of Hsp70, downregulation of its expression has been tested in a mouse model of PD. In this study, direct expression in the SN by recombinant AAV delivery of BAG-5(DARA), a BAG-5 mutant which inhibits wild-type BAG-5 activity, resulted in increased dopaminergic neuron survival (Kalia et al., 2004). Another possible approach to increase Hsp70 function by using gene therapy techniques could be gene silencing by RNA interference to knockdown Hsp70 downregulators. Although these techniques have not been extensively tested for neurodegenerative therapy, they remain a potentially useful tool (Manfredsson et al., 2006).

4.3 Chaperones or regulatory factors delivery using CPPs

Cell penetrating peptides (CPPs) are the most recent approximation that shows promise towards increasing Hsp70 activity within cells. These are peptide motifs that allow cell transduction of macromolecules including functional full-length proteins. The basic domain of the trans-activator of transcription (TAT) from HIV-1 is the best known among CPPs and it has been shown that fusion with TAT allows proteins to penetrate cell membranes of several cell types and even to cross the BBB (Fawell et al., 1994; Schwarze et al., 1999). In the last few years, TAT-Hsp70 transduction has been reported to be neuroprotective against different kinds of stress in cell models (Lai et al., 2005; Nagel et al., 2008) as well as in a MPTP mouse model of PD (Nagel et al., 2008). In addition, transduction of TAT-Hsp40 has also been shown to be cytoprotective against oxidative stress in cells (Kim et al., 2008). Finally, transduction of HSF-1(+)-TAT, an HSF-1 mutant fused to TAT, capable of activating HSR by itself by itself, was demonstrated to induce Hsp70 expression and to

protect cells against heat stress *in vitro* (Hou & Zou, 2009). Although further investigation is needed, CPPs tagging could prove a powerful tool in therapy against neurodegenerative diseases by allowing the efficient transduction of cytoprotective proteins and factors.

5. Conclusions

There is strong experimental support to propose the Hsp70 chaperone system as a key player in pathogenesis and progression of PD. This central role seems to be especially linked to α Syn, although alternative connections between the Hsp70 machinery and different pathogenic mechanisms underlying PD might exist. Up to this point, there is substantial evidence supporting an active role of Hsp70 in well established PD-related processes. First, in the inhibition or modulation of α Syn aggregation pathway that results in the formation of LBs and the suppression of α Syn-mediated toxicity to cells that leads to neurodegeneration. Second, a prominent role in the control of the activity of the UPS machinery, the general protein degradation and disposal system in the cell. And third, a central role in CMA, which handles the lysosomal degradation of selected cytosolic proteins, including α Syn. In addition, emerging mechanisms for Hsp70 in relation to PD include its participation in the CSP α -Hsc70-SGT complex in the neuron, and the increasing attention paid to the UPR and mitochondrial dysfunction processes in PD, both of which rely on Hsp70.

The accumulated studies thus far suggest that Hsp70 chaperone is a highly versatile protein whose anti-aggregation activity seems to involve different interactions and the formation of transient and highly dynamic complexes with various α Syn species, presumably early oligomers and probably monomers, along the aggregation pathway. This activity can be certainly modulated by the presence of nucleotides and by certain co-chaperones, in particular, Hip. Clearly, further *in vitro* and in cell studies with Hsp70 and co-chaperones, to better understand the full molecular mechanism of mammalian Hsp70 in managing α Syn aggregation, are needed.

As a biomarker of PD, Hsp70 seems not to represent a good candidate itself, probably due to its central role in maintaining cellular proteostasis which is perturbed in several amyloidoses and related diseases. However, the Hsp70 co-chaperone Hip might represent a potentially useful biomarker for early diagnosis of PD, although more studies are needed in this direction. Given that PD is a complex disease, a 'complex biomarker' (i.e. composed of various markers) appears to be the only reliable option. Finally, the Hsp70 machinery can be indirectly enhanced by HSF-1 pharmacological activation, which represents one of the most promising therapeutic approaches for treating this complex and highly debilitating disease.

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This book about Parkinson's disease provides a detailed account of etiology and pathophysiology of Parkinson's disease, a complicated neurological condition. Environmental and genetic factors involved in the causation of Parkinson's disease have been discussed in detail. This book can be used by basic scientists as well as researchers. Neuroscience fellows and life science readers can also obtain sufficient information. Beside genetic factors, other pathophysiological aspects of Parkinson's disease have been discussed in detail. Up to date information about the changes in various neurotransmitters, inflammatory responses, oxidative pathways and biomarkers has been described at length. Each section has been written by one or more faculty members of well known academic institutions. Thus, this book brings forth both clinical and basic science aspects of Parkinson's disease.

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