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Alpha-Synuclein and the Immune Response in Parkinson's Disease

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1. Introduction

In the last few years, it has become evident that the immunological component is of central importance in Parkinson's Disease (PD) pathogenesis and progression. This can also certainly be said about the prominent role that the protein α-synuclein (αSyn) is currently believed to play in the pathobiology of this neurodegenerative disorder. Moreover, the multiple mechanisms through which αSyn might be affecting the immune system appear not to be just a consequence of disease progression, but to actively contribute to the delicate balance between neuroprotection and neurotoxicity that ultimately underlies a given stage of disease.

PD is a proteinopathy, whose pathological hallmark is the presence of deposits of aggregated αSyn in intracellular fibrillar inclusions in neurons of the *substantia nigra pars compacta* (SN) of the brain, known as Lewy bodies (LB) (Croisier et al., 2005; Spillantini et al., 1998), and the loss of dopaminergic neurons (Braak et al., 2003). Three missense mutations, A53T, A30P and E46K, as well as multiple copies of the wild-type (Wt) αSyn gene, are linked to early-onset, familial PD (Gasser, 2005; Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Given that αSyn is the major component of LB in both familial and sporadic PD cases (Spillantini et al., 1998), αSyn is considered a critical factor in PD aetiology. Currently, the cellular and molecular mechanisms underlying the pathological actions of αSyn are poorly understood, and the factors contributing to sporadic PD, representing the vast majority of PD cases, are not known.

αSyn, together with β- and γ-synucleins, belong to the family of synucleins, a group of closely related, brain-enriched proteins. This 140 aa-residue protein is largely located in neuronal presynaptic terminals (Kim et al., 2004b) and in the nucleus (Yu et al., 2007). In particular, it is found in the neocortex, hippocampus and SN (Kim et al., 2004b), and in other brain regions, as well as within astrocytes, microglia and oligodendroglia (Austin et al., 2006; Mori et al., 2002; Richter-Landsberg et al., 2000). It is known to interact with a variety of proteins (Jenco et al., 1998; Peng et al., 2005) and lipid membranes (Jo et al., 2000). The physiological functions of αSyn are still being established, but its interaction with pre-

synaptic membranes and lipids suggests a role in the regulation of synaptic vesicle pools including dopamine release control (Perez & Hastings, 2004) and in lipid metabolism (Cabin et al., 2002; Castagnet et al., 2005; Golovko et al., 2009).

Both *in vitro* and *in vivo* in LB, αSyn can self-assemble to form ordered fibrillar aggregates, characterized by a cross β-sheet structure, that are morphologically similar to the amyloid fibrils found in neuritic plaques in Alzheimer´s disease (AD) as well as in deposits associated with other amyloidogenic processes (Chiti & Dobson, 2006). The initial phase of the aggregation process is thought to involve the formation of intermediate oligomers and protofibrillar species which, according to accumulating experimental evidence, can be more toxic to cells than the mature fibrils into which they develop (Bucciantini et al., 2002; Stefani & Dobson, 2003). These and other findings suggest a common structure-linked toxicity among pre-fibrillar species, and propose similar mechanisms contributing to pathogenesis for this group of diseases (Baglioni et al., 2006; Bucciantini et al., 2004). Overall, different hypotheses have been proposed that postulate that αSyn induces a 'gain of toxic function' upon aggregation (Bennett, 2005).

While αSyn is typically considered as an intracellular protein, it has also been found to be normally present in extracellular biological fluids, including human cerebrospinal fluid (CSF) and blood plasma (Borghi et al., 2000; El-Agnaf et al., 2003; El-Agnaf et al., 2006; Lee et al., 2006; Tokuda et al., 2006). However, αSyn levels have been found to be elevated in plasma from PD and multiple system atrophy (MSA) patients relative to age-matched controls (Lee et al., 2006), while lower levels than normal have been detected in CSF from PD patients (Tokuda et al., 2006). On the other hand, two studies by El-Agnaf and colleagues showed an elevated content of oligomeric αSyn spedies present in plasma (El-Agnaf et al., 2006) and post mortem CSF (Tokuda et al., 2010) from PD patients, compared to controls, indicating that changes in the levels and characteristics of extracellular αSyn are associated with the disease (Lee, 2008). Even though membrane permeability from dying cells could be one contributing factor, it has been suggested that vesicle-mediated exocytosis from normal cells is probably the main source of extracellular αSyn (Lee, 2008). By using brain homogenates and neuronal cell cultures, Lee and colleagues (Lee et al., 2005) have shown that both monomeric and aggregated αSyn can be secreted by an unconventional secretory pathway. On the other hand, extracellular αSyn has been shown to be taken up by neuronal and microglial cells in culture, although the nature of the mechanism involved is still controversial (Lee, 2008). In addition, two recent studies have provided strong evidence for a neuron-to-neuron and neuron-to-non-neuronal cell transmission of αSyn aggregates and their associated cytotoxicity, in cellular and mouse models of PD (Danzer et al., 2011; Desplats et al., 2009), highlighting the importance of extracellular αSyn in the pathogenic mechanism of α-synucleinopathies.

2. Neuroinflammation in PD

Another prominent pathological feature of PD brains is the presence of a robust inflammatory response mediated by activated microglia and reactive astrocytes in affected areas of the SN (Glass et al., 2010). Inflammation is the first response of the immune system to pathogens or irritation. In acute conditions, it protects tissue against invading agents and promotes healing. However, a chronic inflammatory state can turn harmful towards the host´s own tissue (Gao & Hong, 2008; Kim & Joh, 2006). Microglia are the resident immunocompetent cells in the brain (Aloisi, 2001), capable of antigen presentation to

lymphocytes (Kreutzberg, 1996) and rapid activation in response to immune insults and invading of PD pathogenesis in the central nervous system (CNS) (Kim & Joh, 2006). As a result of pathogen invasion or tissue damage, microglia switch to an activated phenotype and thereby promote an inflammatory response that serves to further engage the immune system by recruiting other cells to the site of brain lesion, and initiate tissue repair (Glass et al., 2010). However, uncontrolled inflammation may result in production of neurotoxic factors that can be highly detrimental (Gao & Hong, 2008; Glass et al., 2010). Indeed, inflammation in the CNS and sustained overactivation of microglia, i.e. reactive microgliosis, are currently believed to be actively involved in the pathogenesis of various neurodegenerative diseases including PD, AD, multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Gao & Hong, 2008; Glass et al., 2010; Kim & Joh, 2006; Long-Smith et al., 2009).

At present, whether microglial activation ultimately protects or actually exacerbates neuronal loss in the context of PD and other related diseases is still under debate (Delgado & Ganea, 2003; Gao & Hong, 2008; Halliday & Stevens, 2011; Sanchez-Pernaute et al., 2004; Vila et al., 2001; Wu et al., 2002; Wyss-Coray & Mucke, 2002), although the current view favours the second hypothesis. Evidence of microglial attack in PD is supported by findings from epidemiological studies, animal models, and cell culture experiments (McGeer & McGeer, 2008). Epidemiological studies have revealed that taking ibuprofen anti-inflammatory agent regularly is associated with a 35% lower risk of PD (Chen et al., 2005; Chen et al., 2003), supporting the concept that inflammatory attack is contributing to dopaminergic neuronal loss**.** On the other hand, *in vivo* studies show that the specific early up-regulation of SN microglia in PD correlates with disease severity and dopamine terminal loss (Orr et al., 2005; Ouchi et al., 2005). Overall, studies based on animal models and *in vitro* cell culture, indicate that dopaminergic cells are highly sensitive to inflammatory attack (Castano et al., 1998; Fernagut & Chesselet, 2004) and that microglial cells can be strongly activated to mount such an inflammatory response (Austin et al., 2006). Moreover, it has been recently reported that treatment with CSF from PD patients strongly affects cultured microglial cells, resulting in reduced cell growth, morphological changes, as well as increased content and aggregation of αSyn (Schiess et al., 2010). This illustrates how microglia itself, and not only dopaminergic neurons, can be highly affected by the medium in a PD scenario.

3. αSyn-induced microglial activation

The results gathered thus far using the different PD animal models have substantially increased our understanding of PD's pathogenesis by usually providing different but probably complementary information. Thus, while the MPTP mouse model of PD indicates that inflammation in the SN can be self-propagating and leads to progressive neurodegeneration, the αSyn transgenic animal model demonstrates that overexpression of this endogenous protein can certainly provide a powerful source of inflammation (McGeer & McGeer, 2008). Whether microglial activation is essentially caused by the release of aberrant αSyn species to the extracellular space, (Reynolds et al., 2008b; Wersinger & Sidhu, 2006; Zhang et al., 2005), or otherwise, that neuronal death itself drives microglial immune responses in an αSyn-independent manner (Giasson et al., 2000; Mandel et al., 2005; Przedborski et al., 2001), is still under debate. However, there is ample accumulated

evidence pointing at αSyn as the main trigger of microglial activation in PD (Roodveldt et al., 2008). For example, several studies have demonstrated that extracellular and nigral αSyn- containing aggregates are often surrounded by activated microglia or inflammatory mediators in PD brains (McGeer et al., 1988; Yamada et al., 1992), similarly to what has been described for amyloid plaques in AD (Griffin et al., 2006). Moreover, the extent of microglial activation in the SN from PD patients has been found to be correlated with the degree of αSyn accumulation (Croisier et al., 2005) and with increased αSyn levels as evidenced by in vitro (Kim et al., 2009; Klegeris et al., 2008) and in vivo (Lee et al., 2009a) studies, strongly supporting the view that the protein has a major role in phenotypic changes of microglia. Up to this point, a considerable number of *in vivo* studies with animal models of PD that directly link αSyn with microglial activation have been reported. It has been demonstrated in mice that overexpression of αSyn alone (by using adeno-associated virus, AAV) is sufficient to trigger neuroinflammation, involving not only classical microglial activation but also stimulation of adaptive immunity, preceding the appearance of overt neurodegeneration signs (Theodore et al., 2008). In line with this finding, a rat AAV-based model for overexpressing the A53T αSyn variant in the SN revealed dramatic changes in cytoskeletal protein levels and activated microglia-mediated neuroinflammation in the striatum (with increased release levels of IL-1β, IFN-γ, and TNF-α proinflammatory cytokines), well before neuronal loss was evident (Chung et al., 2009). Another recent study using a AAV rat PD model showed that overexpression of Wt αSyn in the SN not only leads to persistent microglia activation, but that depending on the degree of αSyn-induced neurophatology that models either the onset or the late stages of the disease, different microglial responses will occur: upon lower αSyn expression levels where only neurodegeneration occurs, microglia with antigen-presenting capabilities predominate, whereas levels that can induce neuronal cell death correlate with long-term induction of macrophagic microglia (Sanchez-Guajardo et al., 2010), suggesting that microglia may play different roles during disease progression (Sanchez-Guajardo et al., 2010).

Two recent studies have explored the link between neuroinflammation and αSyn dysfunction by lipopolysacharide (LPS) injection in rat (Choi et al., 2010) or mice (Gao et al., 2011), to trigger systemic and brain inflammation. In the first study, the authors observed increased microglia activation and secretion of proinflammatory cytokines as well as greater nitration of proteins including αSyn, in elderly rats, suggesting that an exaggerated neuroinflammatory response that occurs naturally with aging might contribute to αSyn aggregation and dopaminergic neurodegeneration in PD (Choi et al., 2010). In the second study, the authors evaluated dopaminergic neurodegeneration, αSyn pathology and neuroinflammation in Wt and transgenic A53T αSyn-overexpressing mice (Gao et al., 2011). They observed that, while both models initially displayed acute neuroinflammation, only the latter developed persistent neuroinflammation together with chronic progressive degeneration of nigrostriatal dopamine pathway, accumulation of aggregated, nitrated αSyn, and formation of LB (Gao et al., 2011), suggesting that genetic factors and environmental exposures act synergistically to precipitate the development of PD. On the other hand, microglial cells from αSyn-knockout mice have been shown to exhibit a remarkably different morphology compared to Wt cells (Austin et al., 2006), displaying elevated levels of secreted pro-inflammatory cytokines such as TNF-α and IL-6 after activation, indicating that αSyn plays a critical role in modulating the microglial activation state. More recently, the authors have found that microglial activation in this model is

accompanied by increased protein levels of three enzymes involved in lipid-mediated signalling, which suggests a broader function for αSyn in brain physiology beyond synapsis control (Austin et al., 2011).

In the last few years, several *in vitro* studies have focused on the effects of extracellular αSyn on microglial activation. Zhang *et al*. (Zhang et al., 2005) first reported that exogeneous, aggregated αSyn cause activation of microglial cells, which then become toxic towards cultured dopaminergic neurons. Their results indicate that microglial phagocytosis of αSyn and activation of NADPH oxidase, are critical in αSyn-induced microglial activation and neurotoxicity. This finding is highly relevant considering that aggregated αSyn has been shown to be secreted by exocytosis from neuroblastoma and primary neuronal cells (Danzer et al., 2011; Lee et al., 2005), and by stressed neurons (Klegeris et al., 2008). Moreover, following the discovery that αSyn aggregates can be released from neurons and transmitted to neighbouring cells (Desplats et al., 2009), a study has recently shown that αSyn release by SH-SY5Y neuroglioma cells, especially when treated with MPP^+ neurotoxin, are able to activate the inflammatory response in a microglial cell line (Alvarez-Erviti et al., 2011).

Up to this point, research on αSyn-mediated cell response has focused primarily on the effects on neuroinflammation (Benner et al., 2008) or microglial activation (Cookson, 2009; Reynolds et al., 2008a; Thomas et al., 2007; Zhang et al., 2007; Zhang et al., 2005) of αSyn in its aggregated form. Interestingly, Reynolds and coworkers (Reynolds et al., 2008b) have found that nitrated, aggregated αSyn (N-αSyn) has a stronger stimulating effect on microglia than that of nitrated but non-aggregated αSyn. In addition, several investigations have found that N-αSyn, which has been detected in LB of human brains with PD (Giasson et al., 2000) and has been linked to neurodegeneration in PD mouse models (Benner et al., 2008; Gao et al., 2008), induces a neurotoxic inflammatory microglial phenotype that accelerates dopaminergic neuronal loss (Biasini et al., 2004; Thomas et al., 2007; Zhang et al., 2005; Zhou et al., 2005). By integrating genomic and proteomic techniques, Gendelman and colleagues created a fingerprint of microglial cell activation following its interactions with aggregated N-αSyn in cell culture (Reynolds et al., 2008a), indicating that the activation, which was found to be capable of mediating dopaminergic neurotoxicity, is mainly mediated by the NF-κB pathway (Reynolds et al., 2008a). However, whether extracellular αSyn contains the same modifications than the protein found in LB (Anderson et al., 2006; Giasson et al., 2000; Hodara et al., 2004), which is a typically pro-oxidative environment, is still uncertain (Lee, 2008).

Over the last few years, certain differential functions for non-aggregated, extracellular aSyn in glia have been reported. It has been observed that, in contrast to the aggregated form, monomeric αSyn enhances microglial phagocytosis (Park et al., 2008). A few investigations that explore the effects of non-aggregated αSyn on the cytokine release profile of potentially relevant cells have been recently done using monocytic (Klegeris et al., 2008) or macrophage (Lee et al., 2009b) cell lines, and primary astrocyte (Klegeris et al., 2006) or microglial (Roodveldt et al., 2010; Su et al., 2009; Su et al., 2008) cultures. Indeed, we have observed a strong innate immune response in primary glial and microglial cell cultures elicited by exogenous, non-aggregated αSyn (Roodveldt et al., 2010). Interestingly, a comparative study using unmodified aSyn has recently shown that exogenous non-aggregated αSyn induces higher TNF-α, IL-1β and ROS release levels than aggregated αSyn in microglia (Lee et al.,

2010). These and other recent findings point at the importance of exploring the effects on the immune response of aggregated as well as non-aggregated αSyn.

Even though a study using monocytic THP-1 cell line (Klegeris et al., 2008) had shown modest increases in IL-1β and TNF-α secretion levels after stimulation with A30P, A53T, or E46K αSyn mutants compared to the Wt protein, there is a lack of a comprehensive study of the effect exerted by non-aggregated αSyn, performed with primary cell cultures. With this in mind, we analysed the cytokine release profile of primary microglial cultures —which represesnts a more comparable physiological environment— after stimulation with Wt or the PD-linked αSyn mutants (Roodveldt et al., 2010). Indeed, we found remarkable differences between the αSyn variants in the interleukin and chemokine release profiles and significant effects on the microglial phagocytic capacity (Roodveldt et al., 2010). In particular, we observed marked differences in IL-6 and IL-1β pro-inflammtory cytokines, IL-10 immunoregulatory cytokine, as well as IP-10/CXCL10, RANTES/CCL5, MCP-1/CCL2 and MIP-1α/CCL3 chemokines release levels. Our results indicate that extracellular, non-aggregated Wt αSyn produces a moderate to low pro-inflammatory response in glia, together with a reduction of the immunoregulatory response, and a moderate stimulation of Th1 chemokine secretion. The A30P and E46K pathological variants, on the other hand, can induce strong pro-inflammatory and immunoregulatory responses, together with marked increases in chemokine release levels. This exacerbated innate immune response might explain the earlier onset and more rapid evolution of these two genetic cases of PD as compared to the sporadic kind. Intriguingly, our results from the pathologically-linked A53T variant showed not to provoke a significant innate immune response, which might suggest that other neurodegeneration mechanisms contributing to the pathogenesis of PD, probably involving the adaptive immune response, might exist in this case. Combined with the effect on microglial phagocytosis, our results indicate that these αSyn-induced phenotypes might reflect either a classical (A30P and E36K) or an alternative (A53T) microglial activation state, or a hybrid phenotype (Wt), which could probably explain the different disease progression modes that can occur in PD. Alternative activation of macrophages and microglia is a response to tissue injury that is thought to be involved in tissue repair and restoration (Ponomarev et al., 2007), and has been suggested to play a role in repair and extracellular matrix remodelling in AD (Colton et al., 2006). Currently, there is no other indication that such an activation mode could be operating in the context of PD.

Upon activation, microglia and astrocytes start secreting inflammatory cytokines in order to communicate and mount the immune response to counteract disease or injury. The cytokines TNF-α, IL-1β, IL-2, IL-4, IL-6, TGF-α, TGF-β1, TGF-β2 have all been reported to be increased in the nigrostriatal region and CSF of patients with PD or DLB (Croisier & Graeber, 2006). As a result of αSyn-induced activation of microglia *in vitro*, a few cytokines and metabolites have been shown to be significantly up-regulated (reviewed in (Roodveldt et al., 2008)): IL-6, IL-1β, ICAM-1, TNF-α, IFN-γ, MCP-1, O_2^-, iROS, and PEG_2, glutamate, and iCys. In general, disease-linked αSyn variants show a stronger effect on cytokines release than does the Wt protein. Interestingly, analysis of the microglia transcriptome by Gendelman and coworkers (Reynolds et al., 2008a) after stimulation with aggregated N-αSyn, revealed a significant up-regulation of the toll-like receptor 2 (TLR-2) gene**.** TLRs are known to sense the molecular signatures of microbial pathogens, and play a fundamental role in innate immune responses, inducing the expression of diverse inflammatory genes

(Kawai & Akira, 2007). Therefore, it seems plausible that cells challenged with αSyn, or with certain forms of αSyn, could become hyper-responsive to inflammatory signals.

Activated microglia can also produce substantial amounts of superoxide radicals, which may be the major source of the oxidative stress thought to be largely responsible for dopaminergic cell death in PD. The generation of ROS by microglia activated by αSyn (Thomas et al., 2007) can result in oxidation and nitration of proteins, DNA modifications, and lipid peroxidation, leading to neurotoxicity (Zhang et al., 2005). Oxidation (Ko et al., 2000; Souza et al., 2000) and nitration (Giasson et al., 2000; Souza et al., 2000) of αSyn, in turn, can lead to the formation of more aggregates, which could result in increased cytotoxic effects. Consistent with this, Kelly *et al.* have shown that high levels of oxidized cholesterol metabolites in brains from PD and dementia with LB patients, accelerate αSyn fibrillation (Bosco et al., 2006). On the other hand, McGeer and colleagues (Klegeris et al., 2007a) have reported that αSyn-stimulated microglia, in combination with IFN-γ, produce and increase in the toxicity on human monocytic cells exerted by neurotoxic secretions (Klegeris et al., 2007a). Interestingly, this toxicity can be diminished with specific ligands for ryanodine receptors (Klegeris et al., 2007a), which are known to help mediate the efflux of Ca^{2+} ions from intracellular stores and avoid uncontrolled increases in $[Ca^{2+}]_i$ that may lead to cell death (Giorgi et al., 2008).

Further insight into the mechanism of pathogenesis might derive from the findings that several proteins which are thought to be linked to PD are up-regulated as a result of αSyn-induced microglial activation. Gendelman and co-workers, by determining the activated microglia proteome profile (Reynolds et al., 2008a), found that aggregated N-αSyn activation of microglia results in differential expression of several proteins. These range from proteins involved in oxidative stress, cell adhesion, glycolysis, growth control, and migration, to cytoskeletal proteins. It is worth noting that two of those proteins found to be most up-regulated, calmodulin and ubiquitin, have been shown to interact with αSyn with possible functional consequences. Calmodulin has been shown, *in vitro*, to bind to αSyn in a Ca^{2+}-dependent manner (Lee et al., 2002) and to inhibit αSyn fibrillation (Martinez et al., 2003). On the other hand, a fraction of αSyn found in LB is mono-ubiquitinated (Hasegawa et al., 2002; Tofaris et al., 2003), but the role of this modification remains unclear. Recently, it has been demonstrated that the ubiquitin-protein isopeptide ligase, seven in absentia homolog (SIAH), directly interacts with and monoubiquitinates αSyn, promoting its aggregation (Lee et al., 2008; Rott et al., 2008) and apoptosis (Lee et al., 2008). In addition, there is also evidence implicating a role for the ubiquitin-proteasome system (UPS) in PD (reviewed in (Lim & Tan, 2007)). Also of interest are the elevated levels of Hsp70 observed upon microglial activation. This central chaperone has been demonstrated to inhibit αSyn aggregation *in vitro* (Dedmon et al., 2005; Huang et al., 2006; Roodveldt et al., 2009), in neuroglioma cells (Klucken et al., 2004) as well as in fly (Auluck et al., 2002) and mouse (Klucken et al., 2004) models of PD, protecting cells from the cytotoxic effects of aggregates.

4. Links between αSyn and astrocytes and oligodendrocytes

Together with microglial cells, astrocytes and oligodendrocytes are part of glia, which normally serve neuroprotective functions but, given adverse stimulation as discussed before, they may contribute to develop chronic neuroinflammation (Halliday & Stevens, 2011; McGeer & McGeer, 2008). Compared to microglia, the functions of astrocytes are

poorly understood. Because they have been shown to produce both pro-inflammatory and anti-inflammatory agents, these cells are thought to have a dual role (McGeer & McGeer, 2008). Many ICAM-1-positive astrocytes are seen in the SN of PD brains and this may attract reactive microglia to the area since microglia carry the counter receptor LFA-1 (Miklossy et al., 2006). Indeed, αSyn has been shown to be capable of both of activating microglia and stimulating astrocytes to produce IL-6 and ICAM-1 (Klegeris et al., 2006). On the other hand, astrocytes have been shown to secrete a number of neurotrophic factors that protect dopaminergic neurons in some models of PD (McGeer & McGeer, 2008), but the mechanisms underlying most of these functions are not yet known. Astrocytes have been shown to express αSyn (Tanji et al., 2001). Interestingly, the presence of αSyn-containing inclusion bodies in astrocytes of sporadic PD brains has been observed (Braak et al., 2007; Terada et al., 2003; Wakabayashi et al., 2000). Finally, a recent study showed that astrocyte expression of A53T αSyn leads to the development of progressed paralysis, strong microglial activation, and neurodegeneration (Gu et al., 2010).

There is still little data on the role of oligodendrocytes in PD. αSyn-containing inclusions have been detected in this cell type in MSA, in DLB, and in PD (Campbell et al., 2001; Wakabayashi et al., 2000). McGeer and colleagues have reported the presence of complement-activated oligodendrocytes in the SN of PD cases (Yamada et al., 1992). Intriguingly, transgenic mice overexpressing Wt αSyn in oligodendrocytes have been observed to develop severe neurological alterations and neurodegeneration (Shults et al., 2005; Yazawa et al., 2005), drawing the attention to a possible role of these glial cells in PD.

5. Expression of αSyn by immunocompetent cells

Given that αSyn expression has been reported also in non-neuronal cells, it is currently thought to play a role besides dopamine release control. While searching for a link between the CNS and peripheral immune system in PD, Kim *et al.* (Kim et al., 2004a) found that αSyn was up-regulated in peripheral blood mononuclear cells (PBMC) at the gene level, in idiopathic PD *vs.* non-PD controls. Moreover, by *in vitro* transfection with Wt, A30P and A53T αSyn genes, they found that αSyn expression is correlated to glucocorticoid-sensitive apoptosis, possibly caused by the enhanced expression of glucocorticoid receptor (GR), caspase activation, CD95 (Fas) up-regulation, and ROS production. However, the increase in ROS production by overexpression of the αSyn mutants was markedly greater than for Wt αSyn. αSyn expression has also been found in cultured human macrophages (Tanji et al., 2002) and its expression levels have been reported to increase by stimulation with pro-inflammatory cytokine IL-1β or LPS (Tanji et al., 2002), further supporting a role for αSyn in the inflammatory process. Finally, expression of αSyn in cultured human T cells, B cells, natural killer (NK) cells and in monocytes/macrophages, have been reported (Shin et al., 2000). Currently, it is not known whether expression or aggregation, of αSyn in T cells can be regulated by ligand activation of the T cell. This may be relevant as it could represent a key link between regulation of the adaptive immunity and Syn expression levels.

6. αSyn and the adaptive immune response in PD

In the last few years, mounting evidence has pointed at a possible participation of the adaptive immune system in PD pathogenesis. However, whether this immune response

actually contributes to neurodegeneration, and in that case by which mechanism, remains unknown. The initial observations in PD patients that a small amount of $CD8^+$ T-lymphocytes occur in proximity to degenerating nigral neurons (McGeer et al., 1988), and the occurrence in LB of components of the classical or antibody-triggered complement cascade (Yamada et al., 1992) had suggested a possible involvement of the adaptive immunity in the PD process. More recently, the finding of accumulated IgG in the SN of PD patients and increased expression of IgG-binding receptors on activated microglia (Orr et al., 2005), and the detection of anti-αSyn autoantibodies (AAb) in blood serum of PD patients (Papachroni et al., 2007), suggest that the pathological process may involve adaptive immune-mediated mechanisms. In addition, the observation that humoral immune mechanisms can trigger microglial-mediated neuronal injury in animal models of PD (He et al., 2002), and the finding by Standaert and colleagues of IgG deposition in mouse brains following AAV-mediated αSyn overexpression in the SN (Theodore et al., 2008), further support a role of the adaptive immune system in disease progression.

A possible consequence of the initial microglial activation in the affected regions of PD brains is the local permeabilization of the blood-brain barrier (BBB), leading to infiltration to the affected regions by B- and/or T-lymphocytes (Racke et al., 2000). Indeed, a remarkable T- and B-cell infiltration into the SN linked to αSyn overexpression was observed at the early stages, i.e. before the appearance of significant dopaminergic neuronal loss, reaching levels in the SN of up to 10-fold and 5-fold compared to controls (Theodore et al., 2008). A recent study by Hunot and colleagues (Brochard et al., 2009) has shown that $CD8^+$ and $CD4^+$ T-cells, but not B-cells, had invaded the brain in PD patients and in MPTP-treated mice during the course of neural degeneration. Furthermore, based on these results the authors propose that T-cell dependant toxicity is essentially mediated by $CD4^+$ T-cells and requires the expression of FasL (Brochard et al., 2009). Given that the FasL pathway had been shown to produce proinflammatory cytokine responses in macrophages (Park et al., 2003), the authors speculate that the $CD4^+$ Th FasL-mediated activation of microglia could participate in neuroinflammation and neurodegeneration processes in PD (Brochard et al., 2009).

Based on results obtained with an MPTP murine model of the disease, Gendelman and colleagues (Reynolds et al., 2010) have suggested that the αSyn-specific regulatory T-cells (Treg cells), which are regulatory components of the adaptive immunity, might be able to counteract the autoaggresive effector T-cell responses that exacerbate neuroinflammation (Benner et al., 2008), and therefore contribute to attenuate neurodegeneration in PD. Indeed, the same group has reported that microglial cells stimulated with N-αSyn are susceptible of essentially opposing immune regulatory responses by Treg cells ($CD4^+$, $CD245^+$) and effector T-cells ($CD4^+$, $CD25^-$) in culture (Reynolds et al., 2009a). By analysing an array of cytokines released by treated microglia, the authors found that, while the effector T-cell subset exacerbates microglial-mediated inflammation and may induce neurotoxic responses, Treg cells are able to suppress N-αSyn microglial-induced reactive-oxygen species (ROS) and NF-κB activation and are proposed to be neuroprotective (Reynolds et al., 2009a). Furthermore, the study indicates that Treg cells can regulate microglial inflammation by inducing Fas-FasL-mediated apoptosis of N-αSyn-stimulated microglial cells (Reynolds et al., 2009a). By using a proteomic analysis, the authors further showed that these Treg cells can significantly alter the microglial protein expression profile for certain proteins linked to cell metabolism, migration, protein transport and degradation, redox biology, and cytoskeletal and bioenergetic

metabolism, to presumably attenuate the neurotoxic phenotype caused by N-αSyn stimulation (Reynolds et al., 2009b).

Thus far, accumulated data demonstrate that in the MPTP model of PD, misfolded and aggregated αSyn are secreted from neurons, which promotes pro-inflammatory M1-type microglia and cytotoxic T-cells, therefore amplifying neuronal damage. In sporadic human PD, it is currently unkown which factor triggers disease onset, but it has been proposed that under certain circumstances, a similar set of temporal and mechanistic events could transform neuroprotective microglia and T cells into cytotoxic cells, thereby accelerating disease progression (Appel et al., 2010). This way, activated microglia and the cytokine milieu that they generate might promote T-cell differentiation into different cell subsets in the context of PD (Appel et al., 2010). Indeed, it has been shown that M1 (pro-inflammatory) cells promote, whereas M2 (non-inflammatory) cells reduce, $CD4^+$ Th1 cell proliferation and function (Verreck et al., 2004), but also that, conversely, T-cells can dictate microglial pro- or anti-inflammatory phenotypes (Giuliani et al., 2003; Kebir et al., 2007; Mount et al., 2007). Whether microglia dictate the specific T-cell phenotype or otherwise, that T-cells dictate the specific microglial phenotype (i.e. M1 *vs*. M2), is still unknown (Appel et al., 2010). But overall, the communication established between microglia, T cells and neurons seem to indicate that the immune response is not only a consequence of injury, but that it actively contributes to the balance between neuroprotection and neurotoxicity (Appel et al., 2010; Stone et al., 2009).

To analyse the possibility that humoral immunity may play a role in initiating or regulating inflammation, Orr *et al*. (Orr et al., 2005) analysed the association between nigral degeneration and humoral immune markers in brain tissue of patients with idiopathic or genetic PD and controls. All the patients with PD revealed IgG, but not IgM, binding on dopamine neurons. Moreover, the proportion of IgG-immunopositive neurons showed a negative correlation with the degree of cell loss in the SN, and positive correlation with the number of activated microglia. IgG was found to be concentrated at the cell surface of neurons, but also on their LB, and was shown to co-localize with αSyn. These results, in combination with their finding that activated microglia express high-affinity IgG receptors (FcγRI) in both idiopathic and genetic forms of PD, might suggest that the activation of microglia may be induced by neuronal IgG (Orr et al., 2005).

The question regarding the functional importance of antibodies against antigen-specific, disease-associated neuronal proteins still needs to be addressed. It has been demonstrated that an IgG fraction purified from serum of PD patients causes death of dopaminergic neurons *in vivo* following stereotaxic injection in the SN of experimental animals (Chen et al., 1998), and the presence of immunoglobulins in PD brain tissue have been proposed to lead to the targeting of dopaminergic nigral neurons for destruction (Orr et al., 2005). Currently, it remains unknown whether these anti-αSyn AAb are neurotoxic, or on the contrary, they actually have a neuroprotective role, as has been shown in a human αSyn transgenic mouse model of PD (Masliah et al., 2005).

A recent study has assessed the presence of auto-antibodies (AAb) against all three synucleins in the peripheral blood serum of PD patients and healthy controls (Papachroni et al., 2007). While the presence of AAb against β- and γ-Syn showed no association with PD, multi-epitopic AAb against αSyn were detected in 65% of all patients, with a strong correlation with the inherited mode of the disease. In addition, a recent study based on measuring AAb levels against monomeric, oligomeric, and fibrillar αSyn in serum from PD

patients (Gruden et al., 2011), showed that all three AAb specificities reached the highest values after 5-year of disease duration, and subsided in 10-year sufferers. Intriguingly, there was a ca. 15-fold increase in AAb titre values relative to monomeric αSyn (72% of patients), and a ca. 4-fold increase for αSyn oligomers (56% of patients). Moreover, the authors also found a decline in $CD3^+$, $CD4^+$ and CD8+ T-lymphocyte and B-lymphocyte subsets. Based on these results, they suggest that αSyn toxicity elimination by AAb in early PD pathology might be linked with the decline of lymphocyte subsets reflecting the influence of inflammatory and oxidative stress processes (Gruden et al., 2011).

Despite their potential involvement in PD pathogenesis and progression, the role of NK cells in PD has hardly been explored. NK cells are active members of the innate immune system that act as a first-line defence, and also mediate between the innate and adaptive immune systems (Salazar-Mather et al., 1996; Su et al., 2001). Interestingly, a recent study using blood samples from PD patiens indicates that the NK activity increases as the disease advances (Mihara et al., 2008). Moreover, the study also showed that the NK cell content among the total lymphocytes of the PD group was higher than in the control group (Mihara et al., 2008).

7. Prospects for αSyn-based immunotherapy in PD

In addition to its well known importance in the pathogenesis of PD, αSyn is becoming a primary target for preventing or controlling the process of PD. In the late few years, vaccination for treating some neurodegenerative disorders has emerged as a potentially useful approach. Thus far, this avenue has been scarcely explored for PD. Importantly, immunization with αSyn was shown to generate a humoral response in a mouse model of PD (Masliah et al., 2005), producing beneficial albeit modest results on histopathological markers of the disease. On the contrary, using N-αSyn as the immunogen proved to elicit strong antigen-specific effector T cell responses in MPTP-intoxicated mice that caused exacerbated neuroinflammation and neurodegeneration (Benner et al., 2008). This response was further shown to be largely mediated by Th17 cells and causing Treg dysfunction (Reynolds et al., 2010). In addition, the authors demonstrated that Treg cells from mice treated with the neuropeptide VIP, known to promote Treg responses (Delgado et al., 2005; Gonzalez-Rey et al., 2006), can efficiently modulate N-αSyn-generated immunity in MPTP mice and confer neuroprotection (Reynolds et al., 2010), suggesting a possible novel therapeutic avenue for PD.

Given that microglial activation can maintain or even aggravate the disease process, blocking inflammation or shifting the balance between pro-inflammatory and anti-inflammatory states in a controlled manner, offers one of the most promising strategies for developing palliative (and maybe preventive) therapies for PD and related disorders. Epidemiological data have identified the non steroidal anti-inflammatory drug (NSAID) ibuprofen as neuroprotective for PD (Klegeris et al., 2007b). NSAIDs are thought to act on dopamine quinone formation and activation by αSyn of both astrocytes and microglia. On the other hand, Gendelman and colleagues demonstrated that T cells from mice immunized with Copolymer-1 (Cop-1), are able to attenuate microglial responses and lead to neuroprotection in a MPTP mouse model of PD (Benner et al., 2004). This neuroprotective effect was later found to be mediated by the $CD4^+$ type of T cells, suggesting the possible involvement of Treg cells (Laurie et al., 2007). Later work by the

same group confirmed this hypothesis by showing that passive transfer to MPTP mice of activated Treg cells, but not effector T cells, efficiently suppressed microglial activation and afforded neuroprotection (Reynolds et al., 2007), suggesting that the immunomodulating abilities of Treg cells could potentially be utilized as a therapeutic approach against PD (Stone et al., 2009).

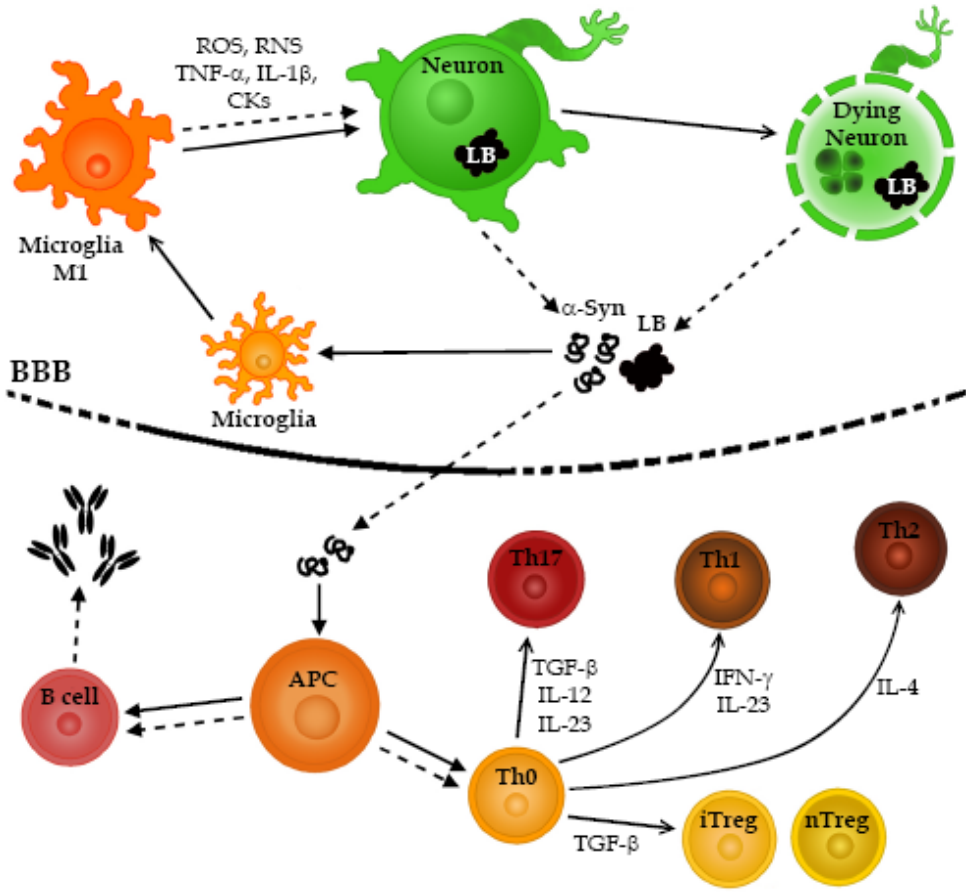

Fig. 1. Links between αSyn and the immune response in PD. Continuous arrows with filled tips: positive effect; continuous arrows with open tips: cell transformation; discontinuous arrows: secretion of cellular factors. APC, Antigen-processing cell; BBB, Blood-brain barrier; CKs, Chemokines; IL, Interleukin; IFN-γ, Interferon γ; LB, Lewy bodies; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; TGF-β, Tumor growth factor β.

8. Conclusion

It is well established that PD onset and progression are characterized by sustained activation of microglia, linked to significant dopaminergic neuron loss in the SN of the brain. Over the last few years, it has become accepted that overexpression and aggregation of αSyn, an amyloid-like

protein, is linked to neurotoxicity through various proposed mechanisms, and may be one of the primary causes of the immunological abnormalities observed in PD. Recent studies with cellular and *in vivo* models of the disease indicate that increased levels of extracellular αSyn, both in its aggregated and non-aggregated forms, are found in a PD scenario.

Accumulated evidence has now established that aggregated extracellular αSyn is able to trigger the activation of microglia, inducing a highly detrimental cascade of neuroinflammation and neuronal demise. In addition, recent studies have have demonstrated that non-aggregated αSyn can also have a substantial impact on microglial phenotype and cytokine release profile, especially in the cases of familial PD αSyn mutants. By releasing toxic factors, or by phagocytosing neighbouring cells, activated microglia and astrocytes are believed to form a self-perpetuating neuronal degeneration cycle. On the other hand, recent findings point at a possible role of the adaptive immune system involving αSyn, and the pathological process in PD. Clearly, further studies in this direction are necessary to help understand the complex immunological mechanisms underlying PD and the key, and possibly multiple, links between αSyn and the immune response in relation to in relation to pathogenesis (Figure 1).

In addition to trying to develop effective tools to prevent αSyn aggregation, modulating the innate immune response by intervening microglial activation, promoting a selective aSyn-specific humoral response, and manipulating the balance between effector and immunomodulatory T-cell populations, may be considered as highly promising therapeutic approaches for the treatment of PD and other synucleinopathies.

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