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REVIEW

Oxidative and nitrative alpha-synuclein modifications and proteostatic stress: implications for disease mechanisms and interventions in synucleinopathies

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Abstract

Alpha-synuclein (ASYN) is a major constituent of the typical protein aggregates observed in several neurodegenerative diseases that are collectively referred to as synucleinopathies. A causal involvement of ASYN in the initiation and progression of neurological diseases is suggested by observations indicating that single-point (e.g., A30P, A53T) or multiplication mutations of the gene encoding for ASYN cause early onset forms of Parkinson's disease (PD). The relative regional specificity of ASYN pathology is still a riddle that cannot be simply explained by its expression pattern. Also, transgenic over-expression of ASYN in mice does not recapitulate the typical dopaminergic neuronal death observed in PD. Thus, additional factors must contribute to ASYN-related toxicity. For instance, synucleinopathies are usually associated with inflammation and elevated levels of oxidative stress in affected brain areas. In turn, these conditions favor oxidative modifications of ASYN. Among these modifications, nitration of tyrosine residues, formation of covalent ASYN dimers, as well as methionine sulfoxidations are prominent examples that are observed in post-mortem PD brain sections. Oxidative modifications can affect ASYN aggregation, as well as its binding to biological membranes. This would affect neurotransmitter recycling, mitochondrial function and dynamics (fission/fusion), ASYN's degradation within a cell and, possibly, the transfer of modified ASYN to adjacent cells. Here, we propose a model on how covalent modifications of ASYN link energy stress, altered proteostasis, and oxidative stress, three major pathogenic processes involved in PD progression. Moreover, we hypothesize that ASYN may act physiologically as a catalytically regenerated scavenger of oxidants in healthy cells, thus performing an important protective role prior to the onset of disease or during aging.

Keywords: aggregation, alpha-synuclein, dopamine, nitric oxide, parkinson's disease, peroxynitrite.

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Abbreviations used: ASYN, alpha-synuclein; CMA, chaperone-mediated autophagy; DA, dopamine; HNE, 4-hydroxy-2-nonenal; MAO, monamine oxidase; MMP, matrix metalloproteinase; NAC, non-amyloid component; NOS, nitric oxide synthase; PD, Parkinson's disease; RNS, reactive nitrogen species; ROS, reactive oxygen species; UPS, ubiquitin-proteasome system.

In a series of neurodegenerative disorders classified as synucleinopathies, such as Parkinson's disease (PD), PD dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and multiple system atrophy, a common feature is the formation and deposition of proteinaceous aggregates, so-called Lewy bodies, and Lewy neurites (Goedert *et al.* 2012; Lashuel *et al.* 2013). A major component of these pathological aggregates is the 140 amino acid pre-synaptic protein alpha-synuclein (ASYN) (Spillantini *et al.* 1997, 1998). Besides the formation of Lewy inclusions, another critical feature of PD is the degeneration of specific neuronal populations, including dopaminergic (DA) neurons of the *substantia nigra* (Hirsch *et al.* 1988). The relationship between ASYN accumulation and neurodegeneration and the molecular basis for the selectivity of this degeneration remain relatively unclear. Several lines of evidence, however, justify our current view that ASYN plays a major role in neurodegenerative processes both in idiopathic and familial PD (McCormack *et al.* 2010). The observation of ASYN as a major constituent of Lewy inclusions hints to its contribution to the onset and progression of the idiopathic disease. Perhaps, more direct evidence of a causal role of the protein in neuronal injury comes from genetic studies. ASYN mutations are a dominant trait for familial PD (Polymeropoulos *et al.* 1996, 1997; Nussbaum and Polymeropoulos 1997). Indeed, point mutations in the ASYN gene, such as the A53T, A30P, or the E46K mutations, were identified in familial cases leading to early onset of parkinsonian symptoms (Polymeropoulos *et al.* 1996, 1997; Krüger *et al.* 1998; Athanassiadou *et al.* 1999; Zarranz *et al.* 2004) including motor impairment. Furthermore, duplications or triplications of the gene encoding for ASYN were identified in familial cases of PD that occurred with an early age of disease onset (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004; Ibáñez *et al.* 2004), suggesting that doubling the concentration of ASYN is sufficient to cause PD. Furthermore, genome wide association studies indicated a link between the ASYN (SNCA) locus and the risk of sporadic Parkinson's disease (Nalls *et al.* 2011).

Studies on the effects of modulating ASYN levels have provided critical insight into the possible physiological function(s) of the protein. Investigations made with animals lacking ASYN revealed some subtle functional deficiencies and suggested that ASYN can act as negative regulator of DA neurotransmission and plays a role in modulating presynaptic vesicle trafficking and brain glucose metabolism (Abeliovich *et al.* 2000; Murphy *et al.* 2000; Michell *et al.* 2007). Physiological regulation of the protein was first described in songbirds, as ASYN was enriched in presynaptic terminals of defined neuron populations during song learning (George *et al.* 1995).

It is important to discriminate between observations of ASYN knockdown, allowing to study physiological functions of ASYN, and observations made with animals over-expressing ASYN, which serve as models to study its pathophysiological functions. Although ASYN-deficient mice exhibit normal development and only subtle functional deficiencies, over-expression of ASYN in different transgenic models reproduces some, but not all aspects of PD pathology (Buchman and Ninkina 2008). Together, these findings suggest that symptoms which are thought to be a consequence of specific neurodegenerative processes (e.g., injury and death of nigrostriatal dopaminergic neurons), may result primarily from a gain of toxic function(s) and possibly partial loss of ASYN's normal functions. The contribution of the latter to the disease pathogenesis may increase with aging.

Other important clues on ASYN pathophysiology came from the elegant work of Braak and colleagues focusing on the spreading of Lewy pathology (Braak *et al.* 1999, 2003; Braak and Braak 2000). ASYN accumulation was shown to begin in the lower brainstem (e.g., dorsal motor nucleus of the vagus nerve) and the olfactory bulb with a subsequent spreading to the pons, midbrain (including the *substantia nigra*) and finally, mesocortical and cortical areas. Two significant corollaries of these observations are (i) the concept of a prion-like spreading of ASYN, and (ii) the view of PD as a 'whole-brain' disease. The latter, however, also emphasizes our need to reconcile what could be perceived as inconsistent observations. On the one hand, ASYN is an abundant protein expressed at relatively high levels (0.5–1% of total neuronal cytosolic protein mass) (Iwai *et al.* 1995) throughout the brain. Pathological changes related to ASYN, such as the formation of Lewy bodies and of other aggregates consisting of this protein, are found in many different brain regions. On the other hand, not all neuronal populations are equally sensitive to the toxic/pathological consequences of ASYN accumulation and aggregation (Luk *et al.* 2012a). A likely explanation for this apparent inconsistency relates to unique features of the susceptible neurons and to a specific ASYN behavior within these cells.

A clear example of such neuron-type selective events is provided by DA neurons in the *substantia nigra*. It has long been known that a pro-oxidant environment characterizes these cells because of their DA content (Fahn and Cohen 1992). When dopamine is released from its acidic storage vesicles either into the synaptic cleft or into the cytosol, it rapidly undergoes enzymatic and non-enzymatic oxidation that yields superoxide, dopamine semiquinone radicals, H_2O_2, and other oxidants (Graham 1978; Fornstedt *et al.* 1990). This feature, together with elevated levels of free iron in the *substantia nigra* (Dexter *et al.* 1989), sets the stage for a Fenton reaction that forms hydroxyl ($^{\bullet}OH$) radicals and could result in sustained oxidative stress (Jenner 1991; Kehrer 2000; Barzilai *et al.* 2003; Arriagada *et al.* 2004). These mechanisms provide a rationale for the high susceptibility of nigral DA neurons to neurodegenerative processes.

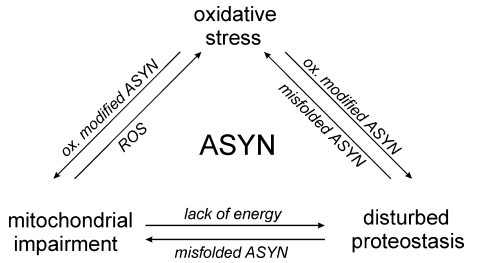

Fig. 1 Alpha-synuclein (ASYN) as link between oxidative stress, mitochondria, and proteostasis.

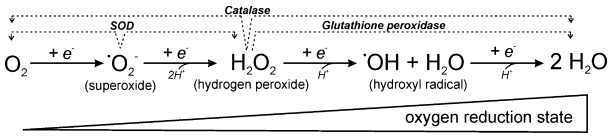

Fig. 2 Formation of reactive oxygen species and mode of action of cellular defense systems. The one-electron reduction of molecular oxygen, mediated, for example, enzymatically by NADPH oxidase, yields superoxide ($^{\bullet}O_2^-$). This can act in a cell as oxidant or as reductant. As selective enzymatic defense systems, the cell contains cytosolic Cu,Zn- or mitochondrial Mn-superoxide dismutase (SOD). Further reduction of $^{\bullet}O_2^-$ yields hydrogen peroxide (H_2O_2). This can either be reduced by cellular glutathione peroxidase or decomposed by catalase to water and oxygen. One-electron reduction of H_2O_2 by Fe^{2+} or Cu^+ yields the highly reactive hydroxyl radical ($^{\bullet}OH$) for which no distinct cellular defense system exists.

They also raise the critical question of whether oxidative stress affects ASYN structure/biology and, if so, whether oxidative damage and ASYN changes could ultimately act together to trigger or promote ASYN-mediated toxic/pathological events in PD (Fig. 1).

In this review, we will highlight the mutual interactions between oxidative stress and ASYN, and the influence of oxidatively modified ASYN on membrane binding, mitochondrial function and proteostasis, as a basis to explain the selective neurodegeneration that characterizes PD and related synucleinopathies. We hypothesize that, at least in certain neuronal populations, modified ASYN might be one of the key 'links' between oxidative stress, proteostatic stress, energy stress, and neurodegeneration. Hence, modified ASYN and the enzymes and/or pathways involved in regulating ASYN modifications could constitute a potential target for preventive or curative intervention strategies.

The large family of reactive oxygen and nitrogen species

The main focus of this review is on oxidative post-translational modifications of ASYN and their impact on the biology of ASYN. Thus, the nature of reactive oxygen species (ROS) and reactive nitrogen species (RNS), their sources in a cell, the sites of formation in the brain, as well as the complex chemistry that is involved, require a thorough discussion. It is essential to note that low levels of free radicals, as observed under normal conditions, do not represent a threat to the cell, and may serve as endogenous signaling molecules, involved in the regulation of physiological processes (Schildknecht *et al.* 2005). These conditions, summarized in the literature under the term 'redox regulation' (Frein *et al.* 2005; Schildknecht and Ullrich 2009) must be clearly separated from conditions of oxidative stress. The basis for our discussion of ROS and RNS is the understanding of the chemical properties of nitric oxide ($^{\bullet}NO$) and superoxide ($^{\bullet}O_2^-$). All other reactive species discussed here are derived from these two free radicals. A one-electron reduction of molecular oxygen (O_2) leads to the formation of superoxide ($^{\bullet}O_2^-$), which can not only act as oxidant but also as strong reducing agent. Further reduction yields hydrogen peroxide (H_2O_2) and finally, the hydroxyl radical ($^{\bullet}OH$) (Fig. 2).

Although $^{\bullet}O_2^-$ is a free radical, its reactivity with biological structures is relatively low in the cellular context and it even acts as a reductant. Another important aspect of $^{\bullet}O_2^-$ is its ionic nature that largely prevents its diffusion across biological membranes at cellular pH. This chemical property has significant biological relevance since $^{\bullet}O_2^-$ is 'trapped' within the subcellular compartment (e.g., mitochondria) where it is formed. At very low pH, $^{\bullet}O_2^-$ may be protonated ($pK_a = 4.8$) and can then cross membranes in the form of its conjugated acid.

H_2O_2 reactivity is relatively weak compared with other ROS such as the $^{\bullet}OH$ radical. Nevertheless, controlling its effective intracellular levels is highly relevant for the survival of a cell. In contrast to $^{\bullet}O_2^-$, H_2O_2 can easily cross biological membranes and together with free ferrous iron, can initiate Fenton reactions and thus produce the hydroxyl radical ($^{\bullet}OH$) (Fenton 1894; Haber and Weiss 1932) (Fig. 3). Among all ROS, $^{\bullet}OH$ displays the highest reactivity toward biological structures. As a consequence of its high reactivity with proteins, lipids, or DNA, it has only a short half-life time and limited diffusion within a cell (Rodebush and Keizer 1947). In contrast to $^{\bullet}O_2^-$ or H_2O_2, no enzymatic degradation systems exist in a cell for $^{\bullet}OH$. Particularly relevant in the context of this review are the following considerations: As already mentioned, DA neurons are at special risk for the toxic consequences of ROS formation and oxidative reactions. For example, within these neurons, H_2O_2 could be generated via both DA autoxidation and monamine oxidase (MAO)-mediated DA metabolism. H_2O_2 could more easily become a substrate for the Fenton reaction because of the high levels of free iron in the *substantia nigra* (Olanow 1992). Finally, a continuous cycle of Haber-Weiss and Fenton reactions could cause substantial oxidation of proteins (including ASYN) and other macromolecules, paving the road to degenerative processes.

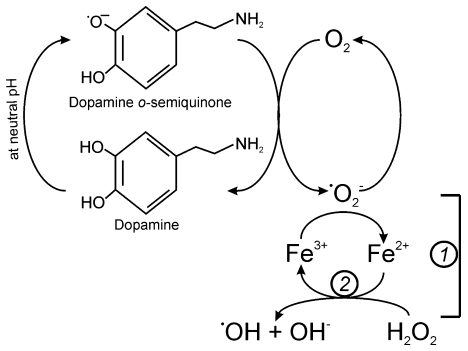

Fig. 3 Hydroxyl radical generation by the Fenton reaction. Dopamine is stable under acidic conditions as observed in neurotransmitter storage vesicles. At neutral pH in the cytosol or extracellular space, it rapidly undergoes autoxidation to form dopamine semiquinones. A redox cycling process of semiquinones and quinones can lead to a continuous generation of $^{\bullet}O_2^-$. This sets the stage for the iron-catalyzed Haber-Weiss cycle (1). This cycle is the driving force for the Fenton reaction (2) that leads to the formation of the highly reactive hydroxyl radical ($^{\bullet}OH$).

Reactive nitrogen species (RNS) are all derived from the small gaseous molecule nitric oxide ($^{\bullet}NO$) that is generated enzymatically from L-arginine in a cell by one of the three isoforms of nitric oxide synthase (NOS). Neurons express NOS-1 (= neuronal, nNOS) that, similar to NOS-3 (= endothelial, eNOS) is characterized by a relatively moderate expression level and cellular activity (Bredt and Snyder 1990; Bredt *et al*. 1990; Radomski *et al.* 1990). In contrast to that, the inducible iNOS (NOS-2) is usually hardly expressed in the brain under normal conditions, but it can be up-regulated significantly in glial cells by a variety of stimuli such as inflammation, or hypoxia (Radomski *et al.* 1990). This isoform leads to the Ca^{2+}-independent formation of high fluxes of $^{\bullet}NO$. The main sources of $^{\bullet}NO$ in inflamed neuronal tissue are microglia and astrocytes (Le *et al.* 2001; Schildknecht *et al.* 2012b). Although nitric oxide is a free radical, its direct reactivity with cellular proteins or lipids is negligible. The interaction between $^{\bullet}NO$ and $^{\bullet}O_2^-$ deserves particular attention in the context of protein modifications. Although both radicals are relatively unreactive alone, the reaction of $^{\bullet}NO$ and $^{\bullet}O_2^-$ to form the peroxynitrite anion ($ONOO^-$) is extremely fast (6.7×10^{-9}/M/s), and even exceeds the dismutation rate of $^{\bullet}O_2^-$ by SOD (2×10^{-9}/M/s) (Beckman and Crow 1993; Huie and Padmaja 1993). This implies that as soon as $^{\bullet}NO$ and $^{\bullet}O_2^-$ are formed within the same cellular compartment, peroxynitrite is generated. This reaction product is a strong oxidant that can react by one-electron oxidations in a radical pathway, and by two-electron oxidations involving oxygen atom transfer. The radical pathway is the dominating source for protein tyrosine nitrations, whereas protein methionine sulfoxidation occurs by oxygen atom transfer (Souza *et al.* 1999). In the discussion of $^{\bullet}NO$ and $^{\bullet}O_2^-$ interaction, it has to be noticed that $^{\bullet}NO$ can freely diffuse across membranes, while $^{\bullet}O_2^-$ has a very limited capacity to cross lipid bilayers. This implies that the localization where $^{\bullet}O_2^-$ is formed determines to a large extent the formation of peroxynitrite and consequently the likelihood for tyrosine nitrations in subcellular compartments. To avoid confusion regarding the designations of different modifications, it is important to distinguish between (i) nitrosylation reactions, in which binding of $^{\bullet}NO$ to a transition metal in an active site center, such as guanylyl cyclase, takes place (Arnold *et al.* 1977); (ii) nitration reactions that mostly modify tyrosine residues with a covalently bound NO_2 group (Tyr-NO_2) (van der Vliet *et al.* 1995), and (iii) nitrosation reactions that, in a cell, mostly modify cysteine residues with a covalently bound NO group (Cys-NO) (Daiber *et al.* 2009; Ullrich and Schildknecht 2012). Important to note, human ASYN contains no cysteines.

Methionine sulfoxidation of ASYN

Oxidation of methionines has been observed as a prominent post-translational modification in several proteins for a long time. In contrast to cysteines, methionines are usually not directly involved in catalytic centers of enzymes. The regulatory role of methionine oxidation is rather associated with structural changes of the respective proteins upon modification. ASYN contains four methionine residues (Met_1, Met_5, Met_{116}, Met_{127}), that can be directly oxidized, for example, by H_2O_2, peroxynitrite, $^{\bullet}O_2^-$, or $^{\bullet}OH$ (Fig. 4). Methionine sulfoxidation of ASYN proceeds sequentially. First, Met_5 is oxidized, while Met_1, Met_{116}, and Met_{127} appear to be more protected from oxidation (Zhou *et al.* 2010). This differential susceptibility may be explained either by the presence of temporary secondary/tertiary structures in the mostly unstructured soluble fraction of ASYN, or by the effect of neighboring amino acids. More detailed studies on the functional and biochemical consequences of ASYN methionine oxidation have been prevented by several technical limitations: chemical oxidative modifications of ASYN always lead to a heterogeneous mixture. Not only the different methionines can take different oxidation states but also other amino acids may be oxidatively modified by the methods used in the past. To circumvent these limitations, new chemical protein synthesis and semisynthetic approaches were very recently developed that allow the site-specific introduction of modified amino acids (Hejjaoui *et al.* 2011, 2012; Butterfield *et al.* 2012; Fauvet *et al.* 2012a). Recently, Maltsev *et al.* demonstrated that Met oxidations in ASYN (Met_1 and Met_5) play an important role in regulating ASYN membrane binding and affinity. They also showed that oxidized ASYN is a substrate for methionine sulfoxide reductase (Maltsev *et al.* 2013). By the enzymatic reversibility of its oxidation, ASYN could

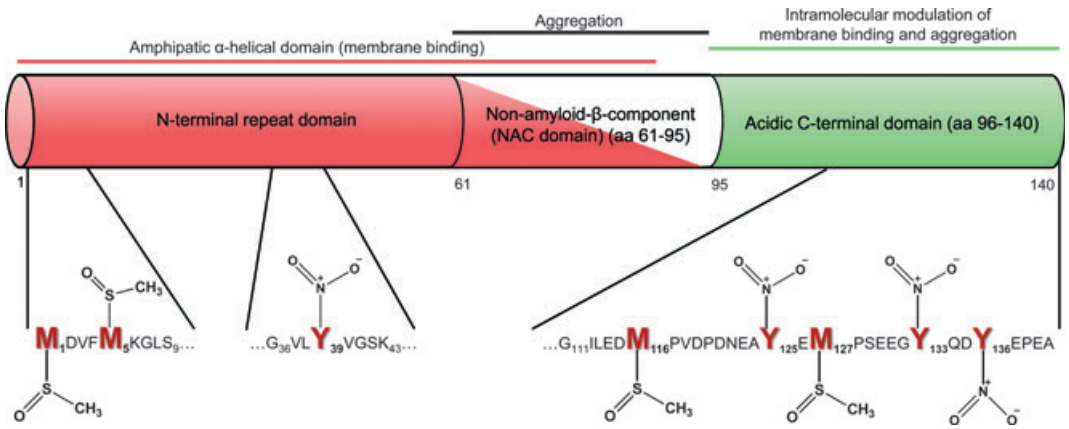

Fig. 4 Oxidative modifications of alpha-synuclein (ASYN). The 140 amino acid protein is composed of an N-terminal domain that adopts an α-helical conformation when in contact to a water-lipid interphase. The central non-amyloid component (NAC) region is mainly involved in the aggregation of the protein, while the acidic C-terminal region has no explicit structural propensity. It serves as regulator of ASYN structure by intramolecular interactions with the N-terminus (George 2002). Highlighted in bold red characters are the four tyrosines (Y), and the four methionines (M) that can be nitrated or sulfoxidated.

contribute to the protection of membranes from oxidative damage.

An overview of the aggregation process and the effect of oxidative modifications is illustrated in Fig. 5. Oxidation of methionine residues in ASYN inhibits its fibrillation by promoting the formation of off-pathway sodium dodecyl sulfate-resistant stable soluble oligomers that no longer contribute to the fibrillation process (Leong *et al.* 2009). These altered aggregation propensities were observed when methionine was oxidized by transition metals (Fe^{2+} or Cu^{+}) or after treatment of the protein with H_2O_2 (Cole *et al.* 2005). The exact reference to the experimental conditions used is important, as the structure of ASYN oligomers that originate upon treatment with different oxidizing agents can vary significantly. For instance, as compared to other toxic oligomers, stable oligomers formed following H_2O_2 treatment did not damage DA or GABAegic neurons (Zhou *et al.* 2010). Given its potential relevance to pathophysiological processes, the nature and toxicity of oligomers formed following methionine oxidation of ASYN warrant further investigation.

Treatment of ASYN with DA or its autoxidation products leads to an inhibition of ASYN fibril formation and a concomitant generation of stable ASYN oligomers (Conway *et al.* 2001; Li *et al.* 2004b; Cappai *et al.* 2005; Norris *et al.* 2005; Bisaglia *et al.* 2010). At least three mechanisms could explain this finding at the molecular level. First, covalently modified ASYN has been observed after DA treatment *in vitro*. However, the low yield, even under optimized experimental *in vitro* conditions, indicates that a significant contribution of this type of interaction under cellular conditions is rather unlikely (Bisaglia *et al.* 2010).

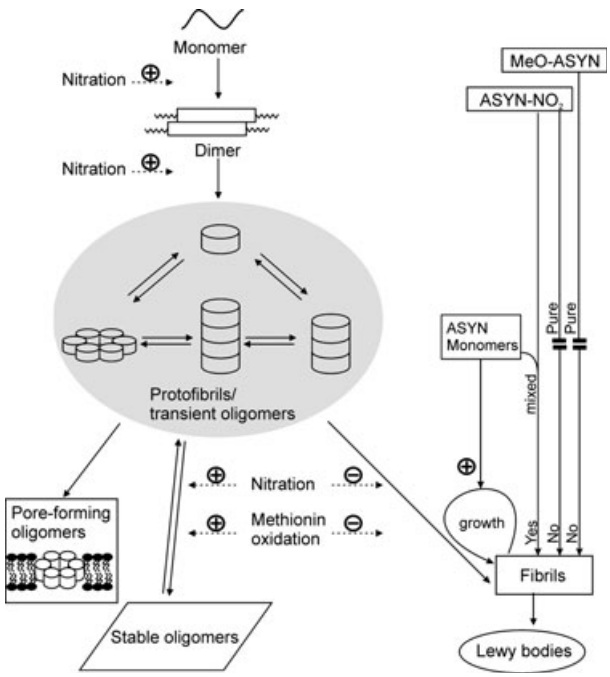

Fig. 5 Regulation of alpha-synuclein (ASYN) aggregation by oxidative modifications. Treatment with peroxynitrite or other oxidants accelerates the formation of covalently linked dimers and/or stable oligomers that no longer contribute to fibrillation. In contrast, unstable oligomers, originating from unmodified ASYN, represent an inhomogenous mixture of spherical, chain-like, or annular protofibrils. Some of these transient structures, for example, can form membrane pores and hence are considered cytotoxic. Nitrated monomeric ASYN alone does not contribute to fibrillation under conditions that favor fibril formation by non-modified ASYN. The situation is different when nitrated ASYN is added to non-modified ASYN. In this case, the modified monomers serve as aggregation seed (right) and accelerate fibrillation.

Alternatively, non-covalent interactions of DA autoxidation products with ASYN could evoke the observed inhibition of fibrillation. Interactions between the aromatic ring of DA (or its oxidation products) with hydrophobic side chains in the C-terminus of ASYN alters the conformation of ASYN and thus promotes the formation of small spherical oligomers (Norris *et al.* 2005; Mazzulli *et al.* 2007). This alternative pathway indirectly prevents the formation of larger aggregates (Herrera *et al.* 2008). DA shares structural elements with a large number of chemically synthesized fibrillation inhibitors, which can undergo oxidation to form quinones. Interestingly, the great majority of these compounds do not only prevent the fibrillization of ASYN, but also of other amyloidogenic proteins, for example, amyloid β peptides (Conway *et al.* 2001; Di Giovanni *et al.* 2010). This suggests that the actions of DA and its degradation products may reflect a more general principle of how this class of molecules regulates protein amyloid formation (Conway *et al.* 2001).

A third mechanism explaining the actions of DA is based on the autoxidation of this neurotransmitter, which generates $^{\bullet}O_2^-$, H_2O_2 and DA semiquinones. These reactive species could directly oxidize methionines into methionine sulfoxides, resulting in an inhibition of ASYN fibril formation (Uversky *et al.* 2002; Hokenson *et al.* 2004; Leong *et al.* 2009; Zhou *et al.* 2009). Mutation of methionine residues to alanine in ASYN blocked the formation of DA-induced SDS-resistant stable soluble oligomers (Leong *et al.* 2009), suggesting that methionine oxidation plays a critical role in DA-mediated oligomer formation. Moreover, it is possible that DA byproducts interact directly with ASYN oligomers and prevent their transition into mature fibrils. What makes the oxidation of methionines unique in comparison with other oxidative modifications, such as nitration of tyrosines, is the existence of a cellular defense system against methionine oxidation. Methionine sulfoxide reductase (Msr) is an enzyme that catalyzes the reduction of oxidized methionines back to their normal state (Yermolaieva *et al.* 2004; Wassef *et al.* 2007; Liu *et al.* 2008) (Fig. 6). Msr is present in two isoforms, that is, Msr A and Msr B, with Msr A being expressed in the nervous tissue. The catalytic activity of methionine sulfoxide reductase within neurons expressing this enzyme makes methionine oxidation a reversible and controlled process (Moskovitz 2005). In fact, one may even speculate that this catalytic function, when applied on ASYN, may confer a role of oxidant scavenger to this protein. Reactive $^{\bullet}OH$, semiquinones and other radical species, when present within the cytosol of neuronal cells, could readily react with methionine residues of ASYN. Then, Msr activity would be capable of repairing this oxidative damage (Maltsev *et al.* 2013). Such a sequence of molecular events, if demonstrated experimentally, would provide evidence for a ROS-scavenging contribution of ASYN under physiological conditions and, possibly, during very initial

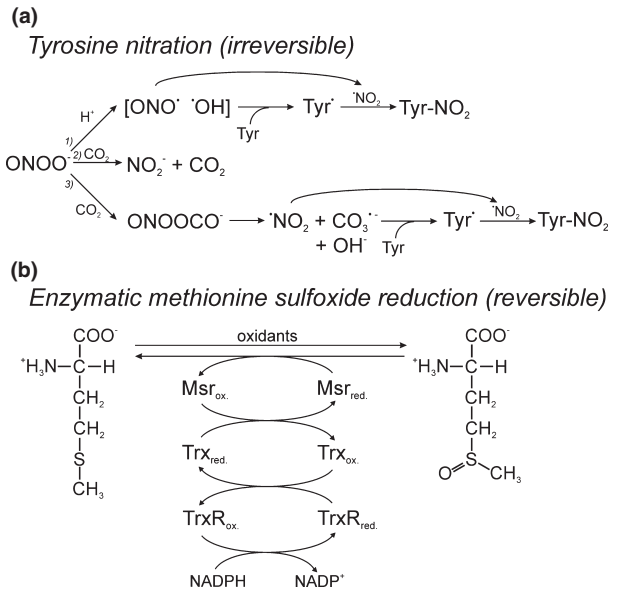

Fig. 6 Chemical biology of tyrosine nitration and methionine sulfoxidation. (a) Tyrosine nitration is an irreversible reaction. At cellular pH, most peroxynitrite is present in its deprotonated form, but peroxynitrous acid (ONOOH) is formed rapidly by protonation (pK_a = 6.6). It is chemically unstable and undergoes homolysis into the $^{\bullet}NO_2$ radical and the hydroxyl radical ($^{\bullet}OH$). The $^{\bullet}OH$ or the $^{\bullet}NO_2$ radical can react with tyrosine residues to form tyrosyl radicals. In the presence of large amounts of bicarbonate, peroxynitrite can also react with CO_2. Two thirds of the resultant reaction product decomposes to yield nitrite (NO_2^-) and CO_2 (2), while about one third generates the $^{\bullet}NO_2$ radical and $CO_3^{\bullet-}$ (3). The $^{\bullet}NO_2$ from (1) and (3) can react with the tyrosyl radical to form 3-nitrotyrosine (3-NT). (b) Methionine oxidation is a biologically reversible reaction. The reduction of methionine sulfoxide is catalyzed by methionine sulfoxide reductase (Msr). This enzyme uses the thioredoxin reductase (TrxR)-thioredoxin (Trx) system, driven by NADPH for its enzymatic oxidation–reduction cycle.

stages of PD. In early PD, at a time when the glia-dependent production of $^{\bullet}NO$-derived reactive species has yet to be induced (see below), the balance between ROS and RNS formation may favor $^{\bullet}O_2^-$ and its derivatives and therefore facilitate ASYN-methionine oxidation/reduction reactions.

Tyrosine nitration and di-tyrosine formation of ASYN

Nitration of protein tyrosine residues is a classical hallmark of most pathophysiological conditions (Duda *et al.* 2000). There are several examples in the literature indicating that nitration can alter the activity and structure of ASYN and other proteins (Giasson *et al.* 2000; Ischiropoulos 2009; Schildknecht *et al.* 2012a). ASYN is a prominent target for peroxynitrite-mediated nitration (Fig. 4) and nitrated ASYN has even been suggested as clinical biomarker for the diagnosis of PD (Fernandez *et al.* 2013). Recent work also

underscores an intriguing relationship between PD risk factors and ASYN nitration. Aging is perhaps the only unequivocal risk factor for idiopathic PD. Interestingly, the *substantia nigra* of the aging primate brain is characterized by enhanced levels of soluble ASYN (Li *et al.* 2004a; Chu and Kordower 2007) and by increased levels of post-translationally modified ASYN, including nitration (Giasson *et al.* 2000; McCormack *et al.* 2012). Another potential PD risk factor is exposure to environmental toxins (Vieregge *et al.* 1988; Di Monte 2003). Experimental models reproducing toxicant-induced injury of nigrostriatal dopaminergic neurons also feature an up-regulation of ASYN as well as formation of nitrated ASYN (McCormack *et al.* 2008). Taken together, these findings suggest that ASYN nitration is a marker of increased neuronal vulnerability to degenerative processes and may itself contribute to pathogenic events underlying human synucleinopathies.

About 20–30% of peroxynitrite ($ONOO^-$) is present in its protonated form peroxynitrous acid (ONOOH) under physiological pH conditions (Goldstein and Czapski 1995; Kissner *et al.* 1997). Peroxynitrous acid dissociates readily into the highly reactive $^{\bullet}OH$ radical and the nitrogen dioxide radical ($^{\bullet}NO_2$) that represents the actual nitrating species (Prütz *et al.* 1985; Merényi *et al.* 1998). A more sophisticated view on the chemistry of peroxynitrite-mediated tyrosine nitrations includes the role of carbon dioxide/bicarbonate that is present in cells in the millimolar concentration range, that is, several orders of magnitude higher than steady-state peroxynitrite levels (Goldstein *et al.* 2001) (Fig. 6). The interaction of $ONOO^-$ and CO_2 has a sufficiently high rate constant (2.9×10^4/M/s) to consider it as one of the dominating pathways for peroxynitrite decomposition in biological systems (Lymar and Hurst 1995; Uppu *et al.* 1996). The intermediate $ONOOCO_2^-$ was shown to decompose partially into $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (Bonini *et al.* 1999; Goldstein *et al.* 2001). While $^{\bullet}NO_2$ is the predominant nitrating species, formation of a tyrosyl radical is a second prerequisite for the occurrence of tyrosine nitration. Formation of tyrosyl radicals through interaction with $^{\bullet}OH$, $CO_3^{\bullet-}$, or $^{\bullet}NO_2$ not only sets the stage for the nitration by the $^{\bullet}NO_2$ radical but alternatively can also lead to the formation of covalent di-tyrosine bonds (Pfeiffer *et al.* 2000; Souza *et al.* 2000) (Fig. 7). At lower peroxynitrite levels, or at higher substrate levels, a shift from tyrosine nitration to tyrosine dimer formation could be expected, as the likelihood for two tyrosyl radicals to encounter each other is increased. The strong concentration-dependence and the short half-life time of its intermediates limit the incidence for di-tyrosine formation for most proteins in a cell. The situation is different for ASYN. Its high abundance in the cytosol would favor di-tyrosine formation between ASYN monomers, and the end-product has been observed both *in vitro* and *in vivo* (Souza *et al.* 2000). The precise position of the respective tyrosines involved in the di-tyrosine formation is still unclear, but preliminary data from our group suggest a preferred role of Y_{39} in di-tyrosine formation. We observed that Tyr_{39} was relatively resistant to nitration (Schildknecht *et al.* 2011), and an ASYN mutant, lacking the three C-terminal tyrosines, was resistant to nitration but rather formed SDS- and heat-stable ASYN dimers (Gerding *et al.*, unpublished). The pathogenic A30P and A53T mutants exhibited an increased propensity for dimer formation via di-tyrosine formation, most likely because of their greater propensity to self-interact (Narhi *et al.* 1999; Kang and Kim 2003). Theoretically, all potential di-tyrosine combinations between two ASYN monomers may be formed. A preference for defined combinations however appears as a more likely scenario since the di-tyrosine formation process is in competition with the nitration of tyrosines. A simultaneous involvement of a single tyrosine residue both in the formation of a di-tyrosine dimer as well as a target for

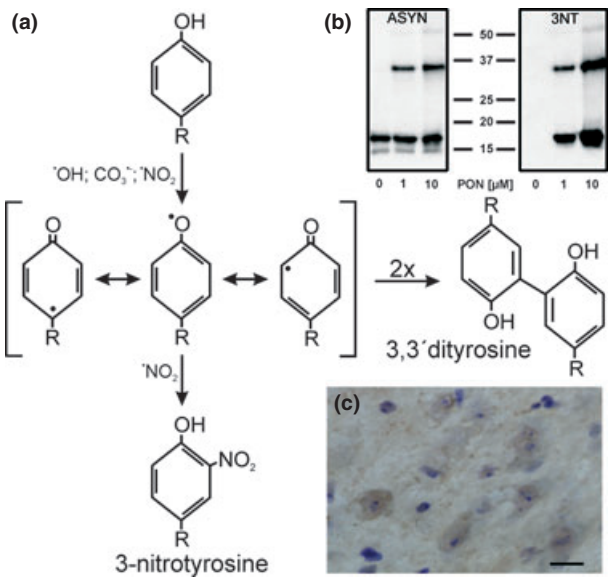

Fig. 7 (a) Mechanism of tyrosine nitration and di-tyrosine formation. Tyrosine is oxidized to form an instable tyrosyl radical by various oxidants. In the presence of the $^{\bullet}NO_2$ radical, nitration via a radical-based mechanism takes place. Alternatively, when two tyrosyl radicals encounter each other, a covalent di-tyrosine bond can be formed. (b) For the western blots, purified alpha-synuclein (ASYN) was treated with peroxynitrite as indicated. The membranes were stained with an anti-ASYN, or with an anti-3-nitrotyrosine (3-NT) antibody. Peroxynitrite caused nitration of the ASYN monomer. In parallel, a second band with the mass of two ASYN monomers appears. This dimer also exhibited tyrosine nitration. (c) Nitrated ASYN is observed in the aging brain (McCormack *et al.* 2012), Parkinson's disease (PD) (Giasson *et al.* 2000), or during experimental neurodegeneration (McCormack *et al.* 2008). As an example, dopaminergic neurons in the *substantia nigra* of a squirrel monkey, treated with MPTP, are shown. Widespread immunoreactivity (brown) for nitrated ASYN is detected. The darker dots within neurons represent neuromelanin. The tissue was obtained from an animal four weeks after a single subcutaneous injection of 1.75 mg/kg MPTP. Scale bar = 10 μm.

nitration cannot be excluded completely, but from a chemical point of view, appears less likely.

Influence of oxidative modifications on the toxicity of ASYN

The central role of ASYN in the pathogenesis of PD and other synucleinopathies raises the question on how oxidative modifications affect its toxicity. The best-characterized mechanisms of ASYN cytotoxicity are associated with the protein's tendency to undergo aggregation. For instance, protofibrillar forms of ASYN were shown to bind to membranes and cause membrane permeabilization via pore-like mechanisms or enhanced flip-flop of membrane lipids (Volles *et al.* 2001; Stöckl *et al.* 2011). Prominent examples are neurotransmitter vesicles that become leaky (Lotharius *et al.* 2002) or mitochondria that display impaired function upon protofibril binding (Hsu *et al.* 2000; Parihar *et al.* 2008; Kamp *et al.* 2010). The processes of aggregation and membrane binding are discussed in detail in the following sections.

Oxidatively modified ASYN has been shown to inhibit not only its own degradation by chaperone-mediated autophagy (CMA), but also that of other proteins (Martinez-Vicente *et al.* 2008; Xilouri *et al.* 2009). As a result of these events, an imbalance between the formation and degradation of such proteins occurs. This does not only result in the accumulation of misfolded proteins but allows an elevation of normal functional proteins. This has for instance been shown for the transcription factor Mef2D (Yang *et al.* 2009). The effect of aggregation-prone proteins on cellular levels of unrelated proteins may be a general principle, that is also known from Huntington's disease, where huntingtin can influence the transcription machinery and hence influence cellular processes (Steffan *et al.* 2000; Nucifora *et al.* 2001; Schaffar *et al.* 2004; Cui *et al.* 2006). Although the cytotoxic mechanisms discussed so far relate to intracellular events, different pathophysiological mechanisms may be associated with extracellular ASYN, excreted from neurons via exocytosis and/or release after neuronal injury (El-Agnaf *et al.* 2006; Tokuda *et al.* 2010; Hansen and Li 2012). Elevated levels of extracellular ASYN initiate microglial activation that in turn leads to the release of pro-inflammatory cytokines (Zhang *et al.* 2005; Lee *et al.* 2010; Fellner *et al.* 2013). Relevant for our discussion on the impact of oxidative modifications of ASYN is the observation that nitrated ASYN can activate microglia via the integrin receptor α5β1 (Liu *et al.* 2011). An inflammatory activation of microglia subsequently leads to a secondary activation of astrocytes (Giulian *et al.* 1994) and both cell types can form relatively large fluxes of ROS and RNS. These free radical species can directly lead to damage of adjacent neurons (Le *et al.* 2001) and trigger the nitration of so far unmodified ASYN (Gao *et al.* 2008). In addition to the direct activation of microglia, ASYN also stimulates the adaptive immunological response (Benner *et al.* 2008; Theodore *et al.* 2008; Stefanova *et al.* 2011). Nitration of ASYN hence leads to a breakage of immunological tolerance because of the generation of a new antigen unknown to the immune system. Nitrated ASYN induces proliferation and activity of specific effector T cells that contribute to the degeneration of DA neurons in the *substantia nigra*. Interestingly, Reynolds *et al.* observed a protective role of regulatory T cells in ASYN-NO_2-mediated adaptive T-cell response. In this study, vasoactive intestinal peptide (VIP) was used as an adjuvant known to boost the regulatory T-cell response. Results suggest that an immune response to ASYN is not necessarily detrimental; vaccination strategies, however, could dampen the adverse effects of an immune response directed toward nitrated ASYN in the brain (Reynolds *et al.* 2010).

Influence of oxidative modifications on membrane binding of ASYN

General interaction of ASYN with membranes

ASYN is an intrinsically disordered protein. While it is considered unstructured in solution, it can undergo conformational changes to form an N-terminal alpha-helical region and an unstructured C-terminal domain when it binds to a water-lipid interface with a negative net charge (Davidson *et al.* 1998; Eliezer *et al.* 2001) (Fig. 8). Most of the initial studies on conformational changes upon lipid binding have been performed with defined artificial vesicles in which lipid composition and diameter can be regulated as desired. These investigations revealed that ASYN preferentially binds to phospholipids with a negatively charged head group such as sphingomyelin, phosphatidylserine, or phosphatidylglycerol.

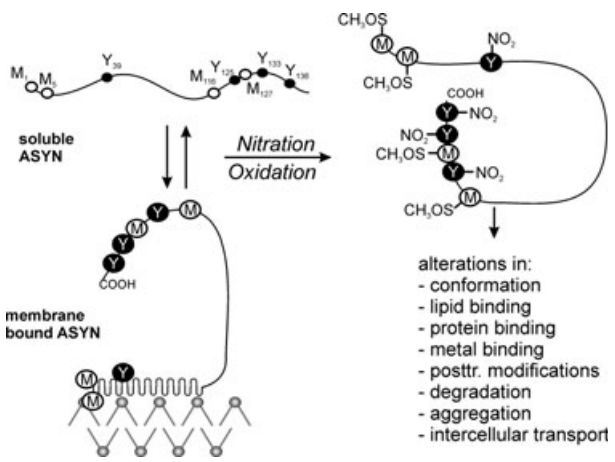

Fig. 8 Influence of nitration on alpha-synuclein (ASYN) biology. ASYN contains four tyrosine residues and is a preferred target for nitration in a cell. Nitrated ASYN displays different properties compared with the unmodified form. These altered properties could contribute to the toxicity of ASYN as observed in various synucleinopathies.

The lipid interaction occurs via the N-terminal region of ASYN (Kubo *et al.* 2005; Beyer 2007). Furthermore, ASYN preferentially interacts with highly curved membranes, as indicated by the observation that intracellular ASYN binds preferentially to small and highly curved structures such as vesicles or mitochondria (Davidson *et al.* 1998; Nuscher *et al.* 2004). When bound to small artificial micelles (diameter ~ 5 nm), ASYN forms a horse-shoe like structure whereas it binds to more physiological lipid vesicles with diameters > 100 nm as an elongated helix parallel to the curved membrane (Jao *et al.* 2004, 2008; Borbat *et al.* 2006). This behavior allows conserved lysine and glutamate residues to interact with dipolar headgroups, while uncharged amino acid residues penetrate into the acyl chain region (Jao *et al.* 2004, 2008). Recently, direct evidence of coexisting horseshoe and extended helix conformations of membrane-bound ASYN has been reported (Robotta *et al.* 2011). At high ASYN/lipid ratios, ASYN is capable of remodeling lipid vesicles, for example, large spherical vesicles can be converted into cylindrical micelles of ~ 50 Å in diameter (Mizuno *et al.* 2012). Rather surprisingly, the outermost 8–10 N-terminal amino acids are absolutely essential for membrane binding, as deletion of this peptide sequence results in a complete loss of membrane binding (Vamvaca *et al.* 2009; Robotta *et al.* 2012). The relevance of the N-terminal region for membrane binding is further underlined by the observation that N-terminal acetylation results in an increase in α-helicity of the first 12 residues when free in solution. N-terminally acetylated ASYN also exhibits increased lipid binding affinity, since pre-formation of an α-helix in aqueous solution significantly increases the on-rate while not significantly affecting the off-rate (Maltsev *et al.* 2012). Interestingly, the A30P, but not A53T mutant has an elevated off-rate from the membrane, compared to the wild-type protein, which is most likely because of the disruption of one of the N-terminal α-helices by the proline (Jensen *et al.* 1998).

Studies using artificial lipid vesicles revealed that membrane binding prevents ASYN from aggregation (Zhu and Fink 2003; Uversky and Eliezer 2009). When ASYN was added to brain membrane preparations, membrane-bound ASYN was identified as seeding nucleus for the aggregation of unbound ASYN monomers (Lee *et al.* 2002). A potential explanation for these apparently discrepant observations could be the presence of cytosolic proteins. Indeed, cytosolic proteins from brain were identified to trigger the dissociation of membrane-bound ASYN (Wislet-Gendebien *et al.* 2006). It could hence be speculated that ASYN aggregation is prevented in systems of artificial vesicles by tight binding of protein monomers to the membranes. In the presence of cytosolic proteins, structural changes in ASYN monomers may occur on the membrane. These could promote the formation of soluble, instable oligomers that are prerequisite for fibril formation.

Alterations in membrane binding through post-translational modifications may play a particularly important role in neuronal cells with a cytosolic ASYN content of up to 0.5–1% (Iwai *et al.* 1995), and with a high surface-to-volume ratio, as in dendrites or axons. For instance, C-terminal phosphorylation does not alter membrane binding (Paleologou *et al.* 2008), whereas phosphorylation at S87 in the central NAC region, interferes with membrane binding (Paleologou *et al.* 2010). Mechanisms by which post-translational oxidative modifications may alter ASYN membrane interactions and therefore contribute to ASYN's pathological role are discussed in detail below.

Influence of oxidative modifications on ASYN membrane binding

Apparently, selective nitration at position Tyr_{39}, that is, within the lipid interaction region, has been shown to decrease ASYN binding to membranes (Hodara *et al.* 2004; Danielson *et al.* 2009) (Fig. 8). The effect may be because of electrostatic repulsion of the negatively charged nitrotyrosine and the negatively charged lipids. As a consequence, nitration would result in elevated levels of free ASYN (Hodara *et al.* 2004). Aggregation of ASYN into cytotoxic protofibrils is dependent on the actual concentration of soluble ASYN (Kim and Lee 2008). Furthermore, nitrated ASYN monomers and dimers were shown to trigger fibrillation of unmodified ASYN (Hodara *et al.* 2004). The decrease of membrane binding by nitration could therefore increase the tendency to form fibrils. N-terminal tyrosine nitration of ASYN would prevent the inhibitory effect that membrane binding has on ASYN aggregation (Narayanan and Scarlata 2001; Zhu and Fink 2003). Interestingly, not only nitration of the N-terminal Tyr_{39} but also nitration of the tyrosines at the C-terminal end (Y_{125}, Y_{133}, Y_{136}) that are positioned outside the membrane binding region, leads to a significant reduction in membrane binding (Sevcsik *et al.* 2011). As explanation, a change in the global structure of ASYN upon nitration that also influences the conformational properties of the N-terminal region was assumed (Sevcsik *et al.* 2011). This observation is of particular relevance with regard to the finding that ASYN nitration at low peroxynitrite levels typically starts at the C-terminal tyrosine residues (Schildknecht *et al.* 2011). Such conditions are likely to be found in inflamed brain tissue with activated glial cells. Hence, it is possible that the reduced membrane binding of nitrated ASYN leads to an increased concentration of free ASYN. This may ultimately result in an increased tendency to form fibrils; however, experimental evidence in cells is currently not yet available.

Regulation of mitochondrial fission and fusion by ASYN

Scientific interest in the interaction of ASYN with biological membranes, lipid metabolism, and mitochondria was aroused by initial findings in yeast models (Outeiro and Lindquist

2003; Willingham *et al.* 2003) showing that some disease-mimicking conditions, such as increasing protein concentrations or introducing the disease mutation A30P, resulted in significant disruption of membrane binding and redistribution of ASYN from the membrane to the cytosol. After it was discovered that ASYN undergoes significant conformational changes when it gets in contact with a water-lipid interface (Jao *et al.* 2004; Ulmer *et al.* 2005), it became obvious that ASYN prefers acidic phospholipids and certain lipid domain structures for optimal binding (Fortin *et al.* 2004). Based on these findings, it was suggested that ASYN is a modulator of synaptic vesicle trafficking and a regulator of vesicle fusion with the pre-synaptic membrane (Lotharius *et al.* 2002). Indeed, mice lacking ASYN, or mice with a knockdown of α-, β-, and γ-synuclein, display an increased DA release that correlated with an increased tendency of neurotransmitter vesicles to fuse with the pre-synaptic membrane (Abeliovich *et al.* 2000; Yavich *et al.* 2004; Anwar *et al.* 2011). In contrast, ASYN over-expression led to an impaired neurotransmitter release and an accumulation of neurotransmitter vesicles at the plasma membrane that were prevented from fusion (Garcia-Reitböck *et al.* 2010; Nemani *et al.* 2010; Scott *et al.* 2010).

It also became evident that the propensity of ASYN to interact with membranes could be relevant to mitochondrial function. A potential physiological role of ASYN in mitochondria is suggested by findings indicating that the 32 N-terminal amino acids of ASYN contain a cryptic mitochondrial targeting signal (Devi *et al.* 2008), as well as by studies in which a complete knockdown of endogenous ASYN expression resulted in an impaired respiratory capacity and an inadequate spatial extension between components of the respiratory chain (Ellis *et al.* 2005). Mitochondrial impairment has long been hypothesized to contribute to PD pathogenesis (Santos and Cardoso 2012). Thus, ASYN-membrane interactions provide an intriguing mechanistic link between ASYN expression/accumulation, mitochondria pathophysiology and PD development.

The morphology of mitochondria is continuously changing in a cell. This does not only involve normal growth and shape changes but also mitochondrial fission and fusion. The latter events are dynamic processes involved in the quality control and maintenance of these organelles (Youle and van der Bliek 2012). In mitochondria, fusion requires the formation of a so-called fusion-stalk, an area with pronounced curvation of the membranes (Nuscher *et al.* 2004; Kamp and Beyer 2006; Kamp *et al.* 2010; Nakamura *et al.* 2011). Over-expression of ASYN results in an increased fragmentation of mitochondria, concomitant with a decline in cellular respiration and ultimately neuronal death (Nakamura *et al.* 2011). The fragmentation observed under these conditions does not require the fission protein Drp1. This suggests a direct interaction of ASYN with mitochondrial membranes (Nakamura *et al.* 2011). In the seminal work of the Haass and Berger laboratories, it was discovered that ASYN preferentially binds to sites of disordered membrane structures that occur under conditions of high curvature, found for example, in synaptic vesicles, but also in the fusion-stalks (Kamp *et al.* 2010; Nakamura *et al.* 2011). By binding to these sites, ASYN seals the packing defects and hereby inhibits the formation of a mitochondrial fusion, ultimately leading to an elevated rate of mitochondrial fragmentation. These observations were further confirmed by a siRNA-mediated knockdown of ASYN, which results in elongated mitochondria (Kamp *et al.* 2010). The described activity may be of pathological relevance, as ASYN-inhibited fusion is restored by the PD-linked genes parkin and PINK1 in their wild-type form, but not by the PD-related mutants (Exner *et al.* 2007, 2012; Lutz *et al.* 2009).

No experimental evidence for the role of oxidative modifications on the regulatory function of ASYN in the fission/fusion process exists so far. For ASYN tyrosine nitration, decreased binding to membranes has been documented (Hodara *et al.* 2004). Based on these findings, it could be speculated that nitration of ASYN might allow higher rates of mitochondrial fusion and favor neurotransmitter vesicle fusion with the pre-synaptic membrane upon stimulation. More work on the interaction of modified ASYN with mitochondria is required, as ASYN not only affects mitochondrial fission and fusion but also can influence respiration by its binding to the inner mitochondrial membrane (Devi *et al.* 2008; Loeb *et al.* 2010). Investigating the impact of oxidative modifications on ASYN's regulatory role in mitochondrial physiology may therefore open a promising field of research on a highly relevant aspect in PD. An effect of ASYN on mitochondrial function may explain why impaired mitochondrial respiration and ATP generation are frequently observed in PD patients (Schapira *et al.* 1990; Arduíno *et al.* 2011).

Effect of oxidative modifications on ASYN aggregation

General aspects on oligomerization and fibrillation of ASYN

Full length ASYN and ASYN-derived peptides were originally discovered as the main components of Lewy bodies in PD and other neurodegenerative diseases (Spillantini *et al.* 1997). These observations *per se* indicate a strong tendency of this protein to form aggregates. Different states and forms of protein aggregation have been described differently in the literature. Here, we will use a combination of recently proposed definitions (Fink 2006; Breydo *et al.* 2012; Lashuel *et al.* 2013). In the beginning of all these pathways, ASYN exists primarily as an unstructured polypeptide chain (Fauvet *et al.* 2012b). In a process that is affected, for example, by temperature, pH, protein concentration, ions, or stirring, the unfolded ASYN undergoes conformational changes to partially adopt a β-sheet structure that is a prerequisite for

fibril formation. (Uversky *et al.* 2001a Uversky *et al.* 2001b) (Fig. 8). A key feature of *in vitro* ASYN fibrillation is a lag phase that is characterized by the formation of transient and unstable oligomers from which monomers can still disassemble (Wood *et al.* 1999). During this lag phase, a critical nucleus needs to be formed first that can then serve as 'seed' for the formation of larger fibrils by the addition of ASYN monomers. Protofibrils, that are routinely described in the literature, are also transient species and represent ASYN oligomers of heterogenous size and morphology distribution. Spherical, chain-like and annular protofibrils have been observed as intermediates during the fibrillization of ASYN *in vitro* (Conway *et al.* 2000; Ding *et al.* 2002; Lashuel *et al.* 2002a, b). In contrast to these highly variable and dynamic oligomeric structures, fibrils are defined as elongated structures with a cross-β-sheet configuration that gain size by addition of monomers to the growing end of the fibril. Because of the flexibility of transient oligomers, monomers can disassemble from these complexes and then contribute to fibril formation. Transient oligomers can furthermore directly contribute to fibril elongation by longitudinal association with a fibril (Fink 2006). In contrast to the formation of transient oligomers from unmodified ASYN monomers, oxidatively modified ASYN monomers can form stable oligomers (Hokenson *et al.* 2004; Uversky *et al.* 2005; Qin *et al.* 2007). Such stable oligomers also contain unmodified monomers and are formed more readily than fibrils under conditions of oxidative stress. Disassembly of ASYN monomers from these stable oligomers only hardly takes place, and consequently, these monomers can no longer contribute to fibril elongation (Uversky *et al.* 2002; Yamin *et al.* 2003; Zhu *et al.* 2004). The formation of stable oligomers, occurring mostly under conditions of oxidative stress, represents therefore an off-pathway redirection of ASYN monomers to oligomer, instead of fibril formation.

Influence of oxidative modifications on ASYN oligomerization and fibrillation

Analysis of ASYN obtained from Lewy bodies of PD brains revealed a wide array of post-translational modifications, including nitration of tyrosines, oxidation of methionines, covalent modification of histidines and lysines by 4-hydroxy-2-nonenal (HNE), and many others, such as phosphorylation, ubiquitination, or SUMOylation. The contribution of such modifications to the aggregation properties of ASYN are an area of intensive investigation.

Nitration of ASYN leads to the stabilization of a partially folded conformation of the monomer and inhibits its fibrillization by stabilizing off-pathway oligomers (Yamin *et al.* 2003; Kaylor *et al.* 2005; Uversky *et al.* 2005). In such oligomers, not only nitrated ASYN monomers but also covalent di-tyrosine cross-links between monomers can be found. This makes the oligomers formed by nitrative insult extremely stable (Souza *et al.* 2000). When reviewing literature data on the nitration of ASYN and its impact on fibrillation, it is essential to discriminate between experimental nitration conditions leading to a mixture of unmodified ASYN, nitrated ASYN monomers, dimers, and oligomers, and studies working with purified nitrated ASYN monomer (Souza *et al.* 2000; Hodara *et al.* 2004). Addition of high concentrations of nitrated ASYN species to unmodified ASYN leads to inhibition of fibrillation. (Uversky *et al.* 2005) (Fig. 5). In contrast, when purified nitrated ASYN monomer or dimer is added in sub-stoichiometric concentrations to unmodified ASYN monomers, the nitrated monomers and dimers, which are characterized by a partially folded conformation, trigger the formation of fibrils from unmodified ASYN by serving as fibrillation seed (Hodara *et al.* 2004). This seeding effect is clearly different from the fibrillation process as such, as purified nitrated ASYN monomers and dimers alone were no longer able to form fibrils (Fig. 5).

Similar to the nitration of tyrosines, methionine sulfoxidation can also affect fibrillation of ASYN. Oxidation of methionines in ASYN prevents the formation of fibrils and instead promotes the formation of stable oligomers (Hokenson *et al.* 2004; Cole *et al.* 2005; Zhou *et al.* 2010). Methionine-oxidized ASYN, when present in excess, can also prevent unmodified ASYN from contributing to the fibrillation process by sequestering the unmodified monomers into the already formed oligomers or poising ASYN oligomer growth and fibril formation (Uversky *et al.* 2002). It is important to note that all data cited above were generated with oxidized ASYN that was used without further purification after the oxidation procedure. Chemical oxidative modifications of ASYN always lead to a heterogeneous mixture of ASYN species with variable modification patterns. Furthermore, treatment with oxidants can lead to the generation of covalently linked ASYN dimers and multimers. So far, no information is available on the aggregation properties of molecularly defined ASYN monomers with oxidized methionines at specific positions. This is mainly because of the lack of methodologies that allow site-specific oxidative modifications of ASYN, a limitation that has been addressed by recent advances made by the development of chemical and semisynthetic strategies for preparing ASYN (Hejjaoui *et al.* 2011, Hejjaoui *et al.* 2012; Fauvet *et al.* 2012b).

Under conditions of oxidative stress, lipid peroxidation takes place and leads to the generation of reactive aldehydes, among them, 4-hydroxy-2-nonenal (HNE), is routinely detected. It is a marker of lipid peroxidation in a variety of experimental and pathological conditions, including neurodegenerative diseases (Yoritaka *et al.* 1996). HNE covalently binds to histidine and lysine residues and triggers the formation of β-sheet rich ASYN oligomers (Bae *et al.* 2013). Similar to the nitration of tyrosines or the oxidation of methionines, HNE modifications prevent fibrillation by

promoting the formation of off-pathway oligomers (Qin *et al.* 2007; Bae *et al.* 2013).

These three examples of oxidative modifications of ASYN illustrate a re-direction of ASYN monomers from the preferred fibrillation pathway into the alternative off-pathway that results in the formation of stable oligomers. Interestingly, the PD mutations have also been shown to promote the formation of off-pathway aggregates in addition to the fibrillization competent ASYN oligomers (Conway *et al.* 2000). Whether the oligomer formation route represents a protective or cytotoxic mechanism, remains to be investigated. Oligomers formed from mutated ASYN are toxic in rodents (Winner *et al.* 2011). It is important to note that the oligomers formed upon oxidative treatment do not represent one defined species but rather a heterogeneous mixture of oligomers of variable sizes and morphologies. This variability, combined with differences in the pattern of oxidation, could explain the difference in terms of toxic properties observed for oligomers prepared under different oxidative conditions; for instance, some reports indicate that methionine-oxidized oligomers are not harmful to neuronal cells (Zhou *et al.* 2010), while other investigations show that HNE-modified oligomers are highly cytotoxic (Qin *et al.* 2007; Näsström *et al.* 2011a).

Influence of oxidative modifications on ASYN degradation

Genetic observations showing a causal association between familial parkinsonism and ASYN multiplication mutations suggest that any molecular or cellular changes (e.g., aging and toxic exposures) that lead to increased ASYN levels could promote deleterious consequences (Ulusoy and Di Monte 2012). The toxic potential of increased ASYN levels also underscores the importance of intraneuronal mechanisms regulating ASYN homeostasis through its synthesis and degradation. Clearance of ASYN can occur via the ubiquitin-proteasome system (UPS) as well as via lysosomal pathways. It has been suggested that the former may be more relevant under physiological conditions, while the latter could play a more prominent role in pathology (Ebrahimi-Fakhari *et al.* 2011, 2012). Such a distinction, however, may not be as clear-cut. Intriguing evidence indicates that oxidative modifications of ASYN can influence its degradation. While normal ASYN monomers can be degraded by the 20S proteasome (Tofaris *et al.* 2003), this process was slowed down significantly by nitration of ASYN (Hodara *et al.* 2004). In addition, ASYN oligomers, generated under nitrating conditions, inhibit proteasome activity (Lindersson *et al.* 2004). Moreover, oxidation/nitration of ASYN can lead to its C-terminal truncation by the proteasome, instead of complete degradation (Mishizen-Eberz *et al.* 2005). Truncated forms of ASYN are frequently found in PD brains; they aggregate much faster than wild-type ASYN and can even act as aggregation seed for normal, unmodified ASYN (Li *et al.* 2005; Ulusoy *et al.* 2010).

ASYN contains a target motif (KFERQ) for chaperone-mediated autophagy (CMA) that allows its recognition by the heat shock cognate protein of 70 kDa (hsc70) and its translocation into lysosomes for degradation (Vogiatzi *et al.* 2008; Mak *et al.* 2010). The seminal work of Cuervo and colleagues (Cuervo *et al.* 2004) also demonstrated that mutant forms of ASYN (A53T and A30P) bind to the CMA lysosomal receptor LAMP-2A but, instead of gaining access into the lysosomal lumen, act as uptake blockers preventing their own degradation and the degradation of other CMA substrates (Cuervo *et al.* 2004). Interestingly, oxidative modifications of wildtype ASYN can induce similar effects; in particular, DA-modified ASYN has been shown to block protein clearance through CMA (Martinez-Vicente *et al.* 2008). One consequence of the inhibition of CMA by modified ASYN is likely to be the induction of macroautophagy. Macroautophagy could also play a critical role under conditions favoring the formation of large aggregates of ASYN, which cannot be cleared via UPS or CMA.

Taken together, experimental data concerning the effects of oxidatively modified ASYN on the UPS and CMA raise the possibility of the following scenario: The formation of nitrated and DA-modified ASYN could lead to impaired UPS and CMA activity that would in turn decrease the clearance of unmodified ASYN. Enhanced ASYN levels would then favor further production of modified ASYN forms, giving rise to a vicious cycle of protein accumulation and oxidation. Neuronal damage may result from this protein load and could be compounded by an enhanced tendency of ASYN to aggregate and an overall blockage of protein degradation pathways.

Intercellular spreading of ASYN – role of oxidative modifications

One of the most interesting developments in recent years of PD research concerns the transmission of ASYN between cells, resulting in a prion-like spreading of the protein (Braak *et al.* 2003; El-Agnaf *et al.* 2003; Kordower *et al.* 2008; Desplats *et al.* 2009). A critical observation was made in PD patients who had received transplants of healthy neurons; after a period of 11–22 years, these transplanted neurons displayed extensive ASYN pathology in the form of Lewy bodies, suggesting spreading of the pathology from the patient to the donor cells (Li *et al.* 2008, 2010). Both exocytosis and uptake of ASYN, as well as spreading of ASYN pathology, have been observed in cell cultures and rodent models (Lee *et al.* 2005; Luk *et al.* 2012a, b). Moreover, ASYN monomers and oligomers are present in the cerebrospinal fluid (Borghi *et al.* 2000). Exocytosis of ASYN was originally interpreted as an alternative

mechanism for a cell to cope with excessive ASYN levels (El-Agnaf *et al.* 2003). At first glance, this appears as an elegant way to lower intracellular ASYN levels. However, three additional aspects have to be taken into account: (i) extracellular ASYN is cleaved by extracellular matrix metalloproteinases (MMP's) (Sung *et al.* 2005) to form truncated, highly aggregation-prone ASYN products; (ii) extracellular ASYN leads to an inflammatory activation of glial cells associated with NOS-2-dependent $^{\bullet}$NO formation that leads to peroxynitrite generation and promotes nitration of ASYN and other proteins (Reynolds *et al.* 2008, 2009); (iii) exocytosed and transferred ASYN serves as aggregation seed for endogenous ASYN in the respective recipient cells (Volpicelli-Daley *et al.* 2011). Both oligomeric ASYN, as well as 4-hydroxy-2-nonenal (HNE) modified ASYN oligomers can serve as seeding-capable species (Danzer *et al.* 2009; Luk *et al.* 2009; Hansen and Li 2012; Bae *et al.* 2013). Similar to observations made with nitrated ASYN, HNE-modified ASYN formes oligomers and is no longer capable of polymerizing into amyloid-like fibrils, but it can trigger fibrillation of unmodified ASYN (Bae *et al.* 2013). According to the Braak hypothesis, Lewy body pathology in idiopathic PD would spread along defined paths from the peripheral to the central nervous system (Braak *et al.* 1999, 2003, 2006; Braak and Braak 2000; Lee *et al.* 2011; Hansen and Li 2012; Pan-Montojo *et al.* 2012). The role of tyrosine nitration and methionine oxidation of ASYN has so far not been investigated with respect to its influence on the spreading of Lewy body pathology. However, recent biochemical data suggest that HNE-modified ASYN could indeed facilitate spreading of Lewy body pathology (Bae *et al.* 2012). The role of other oxidative modifications or post-translational modifications on ASYN's seeding properties remains to be investigated. In these future studies, it will be important to apply methodologies that allow the generation and analysis of site-specificly oxidized and/or post-translationally modified ASYN. This will allow us to dissect the relative contribution of oxidative modifications of each residue and to explore potential cross-talk between the different oxidized residues or different types of oxidative modifications. If earlier observations are corroborated by such more stringent approaches, oxidized ASYN could be a promising target for pharmacological and immunological intervention strategies.

Conclusions and Outlook

Evidence has been compiled here on the occurrence of oxidative modifications of ASYN, the mechanisms involved in this chemical modification process, and the consequences of such modifications on the properties and biological function of the protein. The modifications affect various toxic properties of ASYN and may have different consequences, depending on the cellular context and metabolic conditions. This important concept is clearly illustrated by the following scenario: The C-terminus of ASYN is involved in the binding of metals, such as Cu^{2+} or Fe^{2+}, as well as in the interaction of ASYN with other proteins such as tau. Since several post-translational modifications, including phosphorylation, nitration, and oxidation, occur within the last 20 C-terminal amino acids of ASYN, it is plausible to speculate that these post-translational modifications may act independently or in concert as molecular switches for regulating ASYN interactions with metals, small molecules and proteins. Such effects would be highly dependent on the specific brain region, cell type, and intracellular environment. Furthermore, relatively universal oxidative modifications of ASYN could result in cell/tissue-specific consequences in different types of synucleinopathies and at different disease stages.

Not all neuronal populations are equally susceptible to neurodegenerative processes in synucleinopathies, and the fact that DA cells are among the most vulnerable neurons, strongly suggests an important role of oxidative reactions and oxidatively modified forms of ASYN in their demise. We have highlighted the special situation of dopaminergic neurons. The oxidative environment, characteristic of these neurons (because of their dopamine content) and nigral tissue (susceptibility to neuroinflammation) in PD, provides a conducive environment for oxidative modifications of ASYN. Therefore, the potential gain of toxic function of ASYN caused by its oxidative modifications is likely to be of particular relevance for neurodegenerative processes targeting the nigrostriatal pathway. For instance, methionine oxidation and tyrosine nitration lead to altered aggregation processes. Some ASYN oligomeric species formed under such conditions have been reported to be cytotoxic because of their membrane permeabilizing/disruption properties. This could lead to permeabilization of neurotransmitter vesicles and/or mitochondrial damage, thus contributing to toxic events that have long been associated with nigrostriatal demise in PD.

It is important to emphasize that PD and other synucleinopathies are multifactorial diseases that share similarities but are also characterized by significant clinical and pathological differences. In these diseases, region-specificity and other pathological features would be unlikely to arise from a single molecular initializing event. Rather, mechanistic interactions involving ASYN, oxidative stress, proteostatic stress, and other factors are supposable to take place in the affected cells/tissues (Fig. 1). Specific combinations of toxic events would ultimately lead to disease features unique to PD, multiple system atrophy, or other synucleinopathies. Oxidative modifications of ASYN should be seen more and more within the context of such interactive pathogenetic mechanisms.

In addition to tyrosine nitration and methionine oxidation, several other post-translational modifications of ASYN, such

as ubiquitination, glycation, SUMOylation, or phosphorylation, are subjects of intensive research efforts (Engelender 2008; Oueslati *et al.* 2010; Vicente Miranda and Outeiro 2010; Braithwaite *et al.* 2012). In the future, it will be important to consider the cross-talk between these different modifications and to determine how they regulate each other. For instance, tyrosine nitration may affect phosphorylation of tyrosines. As mentioned earlier, previous studies on the impact of oxidative modifications on ASYN were often hampered by the generation of mixtures of ASYN species with different modification patterns. Recent advances that allow the synthesis of homogeneously and site-specifically modified forms of ASYN will undoubtedly provide more refined insights into the role of oxidative modifications on ASYN's biology (Hejjaoui *et al.* 2011, 2012; Butterfield *et al.* 2012).

Not all post-translational modifications act necessarily as gain of toxic function mechanisms. In fact, loss of normal ASYN function might be sufficient to contribute to neurodegeneration. For instance, as discussed above, it can be hypothesized that normal ASYN plays a role as an antioxidant in the brain. An oxidation–reduction cycle involving ASYN methionine sulfoxidation and methionine sulfoxide reductase activity may protect brain cells when sufficient levels of soluble ASYN are present. Quite in contrast to methionine oxidation, nitration of tyrosine residues seems to be an irreversible ASYN modification of potential toxic relevance. Relatively high levels of $^{\bullet}NO$ are a prerequisite for sufficient $^{\bullet}NO_2$ radical generation and nitration reactions. Such high levels of $^{\bullet}NO$ only originate from the inducible isoform of nitric oxide synthase-2 that is expressed in glial cells upon inflammatory activation (Galea *et al.* 1992; Hewett and Hewett 2012). Nitration of ASYN may therefore only occur during more advanced stages of the degenerative process and perhaps trigger a vicious cycle in which the interaction between ASYN, oxidative stress, and other toxic mechanisms leads to irreversible cell damage.

A final consideration relates to the importance of translating knowledge on ASYN properties, including its oxidative and nitrative modifications, into strategies to prevent its toxic potential. One treatment strategy could be the use of NOS-2 selective inhibitors that are already in development for diseases of the cardiovascular system. Brain NOS-2 inhibition could decrease the irreversible nitration of ASYN and other proteins without interfering with normal redox regulatory processes mediated by basal $^{\bullet}NO$ levels derived from the constitutively expressed NOS-1 and NOS-3. Rodent models using double expression of ASYN and NOS-2 are already available and could be excellent tools for testing potential therapeutics (Stone *et al.* 2012). Since intercellular transfer of unmodified or modified ASYN is likely to play a role in disease progression, immune therapy with specific antibodies represents a promising approach. Indeed, beneficial effects of anti-ASYN antibodies have already been reported in cell culture models (Näsström *et al.* 2011b) and in rodents (Masliah *et al.* 2005; Bae *et al.* 2012). A third pharmacological intervention strategy could target specific ROS sources. One important source of $^{\bullet}O_2^-$ are enzymes of the NADPH oxidase family, such as NOX1 (Chéret *et al.* 2008). Inhibitors of this enzyme, capable of crossing the blood–brain barrier, may be used either alone or in combination with NOS-2 inhibitors and could contribute to a reduction of oxidative protein modifications associated with PD and other neurodegenerative diseases. Future testing of putative protective agents should take advantage of new experimental models of ASYN pathophysiology and human synucleinopathies (Fig. 9). These models include human neural cells that could, for example, be generated from

Burning questions for future research

1. Are different oxidative modifications found in different synucleinopathies?
2. Do different site-specific modifications cause different cellular reactions?
3. Do the type and extent of oxidative modifications correlate with disease stages and progression?
4. Which role do oxidative modifications play on cell to cell spreading of disease and on disease progression?
5. To which extent are different oxidative modifications of ASYN reversible in the brain?
6. Does ASYN act as a scavenger of hydroxyl radicals (antioxidant) in healthy cells?
7. Which is the ideal method for quantifying the extent and site-specificity of oxidative ASYN modifications and the formation of covalent crosslinks in cells and tissues?
8. How do oxidative modifications of ASYN affect other post-translational modifications in the protein?
9. What are the exact mechanisms by which oxidative modifications alter the cellular behaviour of ASYN, including different modes of aggregation, functional interactions with mitochondria, degradation of ASYN and other proteins, binding to lipids and cell organelles and binding to metals or other proteins?
10. Can oxidative modifications of ASYN be used as diagnostic markers or as therapeutic target?

Fig. 9 Burning questions for future research.

patient-derived induced pluripotent stem cells (Liu *et al.* 2012) or from engineered neural precursors (Lotharius *et al.* 2005; Scholz *et al.* 2011, 2013). Animal models in which oxidative modifications of ASYN can be observed, as well as rodents displaying progressive development of ASYN pathology have been described in the literature (Luk *et al.* 2012a; McCormack *et al.* 2012).

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