## **Metallomics**





Cite this: Metallomics, 2015, 7, 395

Received 18th December 2014, Accepted 27th January 2015

DOI: 10.1039/c4mt00339j

www.rsc.org/metallomics

## Insights on the interaction of alpha-synuclein and metals in the pathophysiology of Parkinson's disease

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Parkinson's disease (PD) is the most frequent neurodegenerative movement disorder with severe consequences for patients and caregivers. In the last twenty years of research, alpha-synuclein ( $\alpha$ Syn) emerged as a main regulator of PD pathology, both in genetic and sporadic cases. Most importantly, oligomeric and aggregated species of  $\alpha$ Syn appear to be pathogenic. In addition, transition metals have been implicated in the disease pathogenesis of PD already for decades. The interaction of metals with  $\alpha$ Syn has been shown to trigger the aggregation of this protein. Furthermore, metals can exert cellular toxicity due to their red-ox potential, which leads to the formation of reactive oxygen species, exacerbating the noxious effects of  $\alpha$ Syn. Here we give a brief overview on  $\alpha$ Syn pathology and the role of metals in the brain and then address in more detail the interaction of  $\alpha$ Syn with three disease-relevant transition metals, iron (Fe), copper (Cu) and manganese (Mn). We also discuss possible therapeutic approaches for PD, which are based on these interactions, *e.g.* chelation therapy and anti-oxidative treatments. Not all mechanisms of alpha-synuclein-mediated toxicity and roles of metals are sufficiently understood. We discuss several aspects, which deserve further investigation in order to shed light on the etiopathology of the disease and enable the development of more specific, innovative drugs for the treatment of PD and other synucleinopathies.

Introduction

With the increasing life expectancy, especially in industrialized

countries, there is a growing concern about the rising incidence of

neurodegenerative diseases. The understanding of the etiopathology

and the identification of therapeutic approaches for these disorders

are thus of major public interest in our society.

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#### Minireview

Parkinson's disease (PD) is the second most common neurodegenerative disease and the most frequent neurodegenerative movement disorder inflicting a high personal and socio-economic burden. Within the last two decades, alpha-synuclein emerged as a main regulator of PD pathology, both in genetic and sporadic cases. Transition metals have been implicated in the disease pathogenesis of PD already for almost one century. In spite of these well-known findings, the precise mechanisms of alpha-synuclein-mediated toxicity as well as the role of metals are still not sufficiently understood. This review therefore focuses on recently elucidated interactions of alpha-synuclein and transition metals as major players in the degenerative disease mechanism.

#### Alpha-synuclein and synucleinopathies

Alpha-synuclein ( $\alpha$ Syn) is an highly soluble, intrinsically unfolded protein that has also a high affinity to bind metals.<sup>1</sup> The small 140 amino acid protein, together with beta- and gamma-synuclein, belongs to the family of synucleins and is mainly expressed in the central nervous system (CNS),<sup>2</sup> but also in red blood cells.<sup>3</sup> It is predominantly localized in the cytosol and in the presynaptic terminals in close proximity to synaptic vesicles and has been shown to interact with lipid membranes *in vitro* and *in vivo*.<sup>4</sup>  $\alpha$ Syn has been identified as one of the main components of so-called Lewy-bodies (LB), which are proteinaceous inclusions found in neurons of PD patients and which represent the histological hallmarks of the disease.<sup>5</sup> Since this discovery, the interest for this protein has dramatically increased. Strikingly, LB co-localize with iron<sup>6</sup> and PD patients show altered amounts of metals within the brain,<sup>7</sup> suggesting a role for metals in the etiopathology of the disease.

PD is the most common of the so-called "synucleinopathies" affecting about 1% of the population over 65 years of age.<sup>8</sup> Most cases of PD are sporadic, but rare hereditary forms exist. The first mutation identified was indeed in the gene encoding for  $\alpha$ Syn and nowadays several point mutations (*e.g.* A53T, A30P, E46K, H50Q, G51D) as well as duplications and triplications of the gene have been linked to inherited forms of the disorder.<sup>9</sup> Fibrillar aggregates of  $\alpha$ Syn are also found in other progressive neurodegenerative disorders, *e.g.* in multiple system atrophy (MSA)<sup>10</sup> and in dementia with Lewy bodies (DLB).<sup>5</sup>

Not all of the physiological functions of  $\alpha$ Syn are fully understood. However,  $\alpha$ Syn seems to promote the formation of the soluble NSF attachment protein receptor (SNARE) complex<sup>11</sup> and participates in dopamine (DA) biosynthesis and regulation.<sup>12,13</sup> Interestingly,  $\alpha$ Syn was shown to act as a cellular ferrireductase using Cu and NADH as co-factors to reduce Fe(m) to Fe(n).<sup>14</sup>

Because under physiological conditions  $\alpha$ Syn is poorly structured, it is commonly ascribed as an "intrinsically unfolded protein". However,  $\alpha$ Syn can undergo several modifications of its folding including self-aggregation and fibril formation. The protein comprises 3 parts: a N-terminal part (residues: 1–60), which is mainly a structure that binds to membranes; a central NAC domain (non-Abeta component of Alzheimer's disease amyloid), which has a random coil structure that eventually

misfolds into  $\beta$ -sheets (residues 61–95) and a C-terminal part (residues 96–140), which seems to hinder the fibril formation.<sup>15</sup> The precise mechanisms that induce fibril formation are still unclear. It is believed that the unstructured a Syn monomers shift into a fibrillar structure enriched in β-sheets through various intermediate structural species. Among those species it is possible to find oligomers, pre-fibrils, annular and granular structures.<sup>16,17</sup> One of the most intriguing findings in this regard is that this process can be triggered by the presence of various metals as indicated by biophysical studies. In fact, the presence of Al( $\pi$ ), Cu( $\pi$ ), Cd( $\pi$ ) and Fe( $\pi$ ) has been shown to induce fibrillation.18 Oligomers seem to exert the highest toxicity in vitro and in animal models.<sup>19-22</sup> Their toxicity in vitro has also been attributed to their property to form pore-like structures in the membrane bilayer hence enabling conductance activity bursts. In vivo, oligomers have equally demonstrated to disrupt membranes. Rat brains that were transfected with oligomerprone mutants of  $\alpha$ Syn through injection of lentivirus showed the presence of oligomers and dopaminergic neuron loss three weeks after injection. In contrast, injection of mutants that were more prone to form fibrils showed less toxicity.<sup>20</sup>

One of the most striking hypotheses brought forward in recent years is that a Syn possesses prion-like properties meaning that a misfolded a Syn molecule can impose its folding upon unfolded a Syn molecules and thus contribute to a propagation of pathology. In this mechanism, secreted  $\alpha$ Syn fibrils from the extracellular space act like "seeds" that are capable of recruiting more  $\alpha$ Syn monomers after entering the cell. Subsequently the affected cells release more seeds that further spread the pathology.<sup>23,24</sup> Two main findings support this hypothesis. The first one is the presence of a Syn aggregates in grafted embryonic dopaminergic neurons that has been observed in patient's postmortem brain 14 years after the surgery.<sup>25,26</sup> The second finding supporting the prion-like hypothesis comes from experiments in which pre-formed asyn fibers were taken up from primary cortex neurons. Here, aggregated a Syn was able to recruit endogenous  $\alpha$ Syn, thus forming insoluble aggregates and these  $\alpha$ Syn species were toxic for cells.<sup>27</sup> Despite these observations, this hypothesis remains controversial. In fact, the spread of PD pathology does not follow a nearest neighbor rule.<sup>28</sup>

#### Metals in the brain

The homeostasis of iron, copper and manganese plays a key role in normal brain functions.<sup>29,30</sup> Metals provide positive counterions and, due to their red-ox potential, trace elements are used by enzymes as electron carriers and/or provide catalytic centers for proteins involved in red-ox reactions. An example for this red-ox ability is the detoxifying protein SOD1. In this protein, the catalytic center of Cu( $\pi$ ) is reduced to Cu( $\pi$ ) in the presence of superoxide ions during the process of oxygen radical detoxification.<sup>31</sup>

However, chronic exposure to elevated levels of different metals, *e.g.* copper, zinc, manganese or iron, can also have detrimental effects and induce neurodegeneration. Several metal storage

disorders evidence the development of nervous system pathologies. For instance, Menke's disease or Wilson's disease arise when the Cu metabolism is impaired due to mutations in two genes (ATP7a or ATP7b respectively) that are responsible for intracellular trafficking of Cu and also for its excretion.<sup>32</sup> Impairment in genes that regulate Fe metabolism result in the development of syndromes that are collectively grouped under the name of Neurodegeneration with Brain Iron Accumulation (NBIA).<sup>33</sup> Next to these diseases that originate from defects in metal-regulating proteins, also in PD elevated levels of iron have been observed in specific parts of the brain. PD also shares several clinical features with manganism, a disease that occurs after prolonged, mostly occupational, exposure to manganese.<sup>34</sup>

Furthermore, metals even at low concentrations can readily foster the oligomerization and aggregation of several proteins. Among these proteins we can find amyloid-beta (A $\beta$ ) that oligomerizes with Cu and Zn,<sup>35</sup> amylin that oligomerizes in presence of Cu( $\pi$ ),<sup>36</sup> tau protein that oligomerizes in presence of Al( $\pi$ ) and Fe( $\pi$ ),<sup>37</sup> and of course, also  $\alpha$ Syn can oligomerize in presence of different metals including Al( $\pi$ ), Cu( $\pi$ ), Cu( $\pi$ ) and Fe( $\pi$ ).<sup>18</sup>

One of the pathogenic properties of redox metals is their capability to catalyze the formation of reactive oxygen species (ROS) through Fenton and Haber–Weiss reactions:

$$\mathbf{M}^{n+1} + \mathbf{O}_2^{-\bullet} \to \mathbf{M}^n + \mathbf{O}_2 \tag{1}$$

$$M^{n} + H_{2}O_{2} \rightarrow M^{n+1} + OH^{-} + OH^{\bullet}$$
 Fenton reaction (2)

Net reaction (1) + (2):

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$$
 Haber-Weiss reaction (3)

In these reactions metal ions catalyze the production of highly reactive hydroxyl radicals that are able to oxidize proteins, DNA and lipids, which can result in metabolic impairment and cytotoxicity. Chaperones (like Hsp70) and antioxidants usually counterbalance the production of ROS and their function can be impaired in neurodegeneration.<sup>38-40</sup> Despite their great phenomenological heterogeneity, oxidative stress is considered as a major contributor to the pathomechanism of several neurodegenerative disorders. In fact, there is evidence from Alzheimer's disease,<sup>41</sup> Huntington's disease,<sup>42</sup> Friedreich's ataxia,<sup>43</sup> and amyotrophic lateral sclerosis<sup>44</sup> that high levels of ROS result in neurodegeneration. Oxidative stress appears also to contribute to the death of dopaminergic neurons in PD and therefore several murine models of the disease take advantage of toxins that exacerbate this aspect, i.e. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, 1,1'-dimethyl-4,4'-bipyridinium dichloride (paraquat), and 6-hydroxydopamine (6-OHDA).39

#### $\alpha$ -Synuclein and iron

Iron is the most abundant trace element in the human body. Its abundance is due to its presence in the metalloprotein hemoglobin involved in oxygen-transport, but it has also numerous functions as cofactor in enzymes that require a red-ox counter

ion, e.g. cytochromes, iron-sulfur proteins, catalase or hydrogenase.<sup>45</sup> Like other metals, its metabolism is tightly regulated: when Fe is not bound to proteins, this metal is sequestered in mitochondria and lysosomes. The reason for this highly regulated system is the ability of Fe to actively participate in redox reactions, which has both beneficial and deleterious effects. On one hand iron can be the catalytic center of many enzymes. On the other hand it can foster toxic reactions through the increase of radical species that can damage DNA, proteins and lipids through the Fenton reaction. Therefore, the uptake of Fe is tightly regulated by the presence of the transferrin receptor (TfR) that allows the uptake of Fe from the extracellular milieu where Fe is usually bound to transferrin (Tf); furthermore also the divalent metal ion transporter 1 (DMT1) is thought to import Fe in the cell. On the other hand the export is mediated by ferroportin 1 (FPN1).46

The link between Fe presence and PD has been observed as early as in the 1920s, when Lehermitte and colleagues noticed the co-localization of Fe within LBs using Pearl's staining.<sup>47</sup> The initial studies used mainly histochemical techniques on tissue from patients,<sup>6</sup> but also more sensitive techniques (such as ICP-MS) showed an accumulation of this metal in the *substantia nigra* of PD patients in comparison to controls.<sup>7</sup>

In recent years other very sensitive techniques have been applied to this regard, *e.g.* synchrotron X-ray fluorescence (SXRF). This technique takes advantage of the synchrotron radiation and uses X-ray photons to enable a qualitative and quantitative analysis of chemical elements with a high sensitivity and high spatial resolution. Using SXRF it has been possible to identify an elemental fingerprint of the *substantia nigra pars compacta* of PD patients: for example, the levels of S, Cl, Fe and Zn were significantly different as compared to controls and these variations enabled a cluster separation between these two groups.<sup>48</sup> These studies confirmed previous work using SXRF on paraffin embedded samples, in which there was a significant increase of Fe content in the SNpc of a patient with PD compared to the control patient.<sup>49</sup>

From a biophysical point of view, the direct binding of  $\alpha$ Syn and Fe is rather loose. In fact, studies about  $\alpha$ Syn binding to Fe(II) demonstrated a moderate binding affinity of 50  $\mu$ M measured through tyrosine fluorescence quenching,<sup>50</sup> and more recent NMR studies allowed to infer an interaction of about 1 mM.<sup>51</sup> NMR titration gives evidence that Fe(II) binds  $\alpha$ Syn at the C-terminus at Asp-121, Asn-122, and Glu-123, like other divalent metals.<sup>51</sup> By combination of ESI-MS and cyclic voltammetry the binding constant with Fe(III) was determined to be  $1.2 \times 10^{13}$  M<sup>-1</sup>.<sup>52</sup> Hence a mechanism has been proposed by which Fe(II) associates to  $\alpha$ Syn and in the presence of O<sub>2</sub> the iron oxidizes and there is a dissociation of Fe in the form of Fe(III). Within this reaction H<sub>2</sub>O<sub>2</sub> is produced as a byproduct and this augments the oxidative stress in the cell.<sup>52</sup>

The affinity between  $\alpha$ Syn and divalent metals (including Fe) can vary greatly upon post-translational modification of the protein, such as phosphorylation. This modification is particularly significant for the development of LB, in fact the phosphorylation at Ser-129 is the predominant modification of  $\alpha$ Syn in PD

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patients' brains.<sup>53</sup> Therefore, it is interesting to understand how these modifications can alter the binding properties of  $\alpha$ Syn with metals. The phosphorylation at the C-terminus at Ser-129 or Tyr-125 alters the specificity and binding affinity of metals as demonstrated by electrospray ionization-mass spectrometry (ESI-MS) and fluorescence spectroscopy. These experiments show that upon phosphorylation in those positions  $\alpha$ Syn has increased binding affinity for Cu(n), Pb(n) and Fe(n), but not Fe(m). In addition, phosphorylation at these sites results in a shift of metal binding sites from the N-terminus to the C-terminus<sup>54</sup> (Fig. 1).

Next to the binding affinities between Fe and  $\alpha$ Syn, it has been shown using confocal single molecule fluorescence in combination with atomic force microscopy, that after inducing aSyn oligomer formation in vitro using DMSO in unilamellar vesicles, the addition of Fe(III) results in the formation of larger oligomers that were SDS-resistant. Intriguingly, the subsequent single pore electrophysiology analysis pointed out that these large oligomers could form pores in a lipid planar bilayer.<sup>16</sup> These Fe-induced pores are toxic when applied to cells. Their toxicity is due to the fact that they can interact with the lipid bilayer of the membrane and affect the membrane conductance. Using single channel electrophysiology these aggregates have been shown to act like trans-membrane channels that have some electrophysiological properties similar to those of bacterial porins, e.g. the dependence of pore-conductance on both direction and magnitude of the clamped voltage and the available cation.55

The relationship between  $\alpha$ Syn and Fe has been investigated in regard to the ability of Fe to form reactive oxygen species when the protein is overexpressed. Indeed in BE-M17 neuroblastoma cells overexpressing different  $\alpha$ Syn mutations, the presence of Fe and DA or H<sub>2</sub>O<sub>2</sub> provokes the formation of aggregated  $\alpha$ Syn containing ubiquitin that is LB-like<sup>56</sup> (Fig. 2).

Thus, although both Fe and  $\alpha$ Syn have intrinsic pathogenic properties relevant for the development of PD, their interaction may synergistically foster deleterious mechanisms.

### $\alpha$ -Synuclein and copper

Copper (Cu) is an essential trace element in the brain and it is required for its normal development.<sup>32</sup> It is a cofactor for several cellular proteins, such as cytochrome *c* oxidase (involved in the mitochondrial production of ATP), Cu/Zn-superoxide dismutase (SOD1; that has a role in ROS detoxification) and ceruloplasmin (a blood protein that chelates Cu for its transport throughout the body), just to name the most important ones.<sup>32</sup> Therefore, Cu homeostasis is tightly regulated in the brain. The main proteins involved in the intracellular trafficking of Cu are the copper transporter 1 (Ctr1) and the divalent metal transporter 1 (DMT1) that can import Cu in the cells and the export of this metal is mediated by ATP7a and ATP7b.45 Because this metal is very susceptible to red-ox reactions, the disruption of its homeostasis can be detrimental by causing an increase of oxidative stress in cells. Thus only very small amounts of unbound Cu are usually present in the cell.57

Interestingly, in post mortem brains of PD patients there is a lower Cu concentration compared to controls, especially in the substantia nigra (SN) as measured by ICP-MS<sup>7</sup> and this finding has been recently confirmed by SXRF.58 These data suggest a primary or secondary dysregulation of Cu homeostasis in the brains of PD patients. On the other hand there is also experimental evidence for a direct interaction of Cu and aSyn and for an increased aggregation propensity of a Syn following the binding of Cu. The bond between a Syn and Cu appears to be highly specific and  $\alpha$ Syn is able to bind Cu in the micromolar range.<sup>15,59,60</sup> Two different binding sites for Cu(II) have been identified in the  $\alpha$ Syn sequence through NMR studies: the His-50 site<sup>61</sup> and the Met-1 site.<sup>15,51</sup> Electron Paramagnetic Resonance (EPR) spectroscopy has been applied to further characterize the interaction between  $\alpha$ Syn and Cu(II). These results show that at physiological pH, two binding modes exist: the first one corresponds to the interaction with the Met-1 and Asp-2, while the second one additionally includes binding to His-50. At lower pH of 5.0, the His-50 binding is strongly



Fig. 1 Binding sites of metals to  $\alpha$ Syn. Cu has the highest affinity for  $\alpha$ Syn with two main binding sites at the N-terminus (Met-1 to Met-5 and His-50). When His-50 is replaced by Gln, Cu cannot bind anymore and results in misfolding of  $\alpha$ Syn. Another binding site exists at the C-terminus that has lower affinity, but can bind all divalent metals including Fe(II); Cu(II) and Mn(II). When Tyr-125 and Ser-129 are phosphorylated,  $\alpha$ Syn increases its binding affinities for Fe(II), Cu(II) and Pb(II). For details see text.



**Fig. 2** Metal transport and pathomechanisms involving  $\alpha$ Syn and metal binding. Iron (Fe), copper (Cu) and manganese (Mn) are imported or exported by their respective transport proteins transferrin (Tf), transferrin receptor (TfR), divalent metal transporter 1 (DMT1), ferroportin 1 (Fpn1), copper transporter 1 (Ctr1), copper transporting ATPase 1 (ATP7a), Wilson disease protein (ATP7b), ZRT, IRT-like protein (ZIP), voltage-gated calcium channel (VGCC). Intracellularly, Fe co-localizes with  $\alpha$ Syn-containing Lewy bodies in PD patients' cells. Formation of  $\alpha$ Syn aggregates is fostered by Fe, Cu, Mn and dopamine (DA) and can result in the formation of unstructured aggregates and ion-permeable membrane pores.  $\alpha$ Syn can act as a ferrireductase reducing Fe(III) into Fe(III) when associated to Cu. The chelation of Cu results in a redistribution of  $\alpha$ Syn towards the membrane, while increased Cu levels restore its cytosolic localization and its propensity for aggregation. Mn enhanced the transcriptional and translational  $\alpha$ Syn expression, increasing apoptotic cell death. Ub, ubiquitin; ROS, reactive oxygen species. For details see text.

diminished and  $\alpha$ Syn additionally showed a completely novel mode of Cu( $\pi$ )-binding involving Asp-119, Asp-121 and Glu-123.<sup>62</sup> Mutations in  $\alpha$ Syn responsible for inherited forms of PD (A30P, E46K, A53T) show essentially the same binding to Cu( $\pi$ ), although the A30P mutation favors the second binding mode involving His-50.<sup>63</sup> The aggregation propensity of the same  $\alpha$ Syn mutants was challenged in the presence of Cu( $\pi$ ) and was evaluated through small angle X-ray scattering (SAXS). SAXS revealed that the addition of Cu( $\pi$ ) leads to conformers that were more compact, presumably toxic oligomers, and that the addition of an anti-fibril platinum compound (VK7) broadened the range of Cu( $\pi$ )-induced  $\alpha$ Syn-conformations.<sup>64</sup>

Also Cu(1) can bind directly to  $\alpha$ Syn.<sup>65,66</sup> In fact, two regions have been proven to bind to this metal: the amino acids 1–5 and 116–127. Within the cell, both Cu species co-exist and the transition from Cu(1) to Cu(1) in amyloid aggregates could exacerbate the presence of ROS leading to cell damage.<sup>65</sup> Recently, Cu(1) was shown to even oxidize  $\alpha$ Syn itself.<sup>66</sup> Interestingly, the ability of Cu to bind to  $\alpha$ Syn at His-50 could be highly relevant for disease pathology in human patients as it was recently reported that a single point mutation in the Cu-binding site (H50Q) leads to a familial form of PD.<sup>67</sup> This mutation results in a late onset PD, which shows a rapid disease progression leading to motor impairment and dementia. The molecular basis of this phenotype might be the disruption of the imidazole bond between Cu and His-50 thus enhancing the pathogenicity through a structural modification.<sup>68</sup> This idea is consistent with reports demonstrating a higher fibrillation propensity of the H50Q mutant compared with the wild-type together with the impairment in the Cu bond<sup>69</sup> (Fig. 1).

Being a Cu-binding protein can also be crucial for the physiology of  $\alpha$ Syn. Data obtained with isothermal titration calorimetry suggests that  $\alpha$ Syn can act as a ferri-reductase after saturation with Cu and uses NADH as co-factor to reduce Fe(m) to Fe(n). This activity could also be demonstrated in lysates of cells overexpressing  $\alpha$ Syn.<sup>14</sup>

Cu can contribute to cellular toxicity when associated with  $\alpha$ Syn. For example, in SH-SY5Y cells overexpressing  $\alpha$ Syn, Cu

supplementation increases cytotoxicity.<sup>70</sup> Interestingly, Cu depletion by a chelator results in a redistribution of  $\alpha$ Syn towards the membrane and reduces aggregate formation, while supplementation with CuCl<sub>2</sub> restores its cytosolic localization and its propensity for aggregation.<sup>70</sup> One explanation for this effect could be the ability of Cu to increase the oxidative stress within the cell. In the proposed model, Cu(n) bound to  $\alpha$ Syn is reduced to Cu(i) leading to the production of H<sub>2</sub>O<sub>2</sub>, which in turn can oxidize the neurotransmitter dopamine (DA). Addition of Cu(n)- $\alpha$ Syn complexes to SH-SY5Y cells thus resulted in reduced viability.<sup>71</sup> The role of DA as a third party in the relation of Cu and  $\alpha$ Syn is very intriguing, most importantly because dopaminergic neurons show an increased susceptibility to degenerate in PD.

When the interaction between  $\alpha$ Syn, DA and Cu is evaluated by nanopore analysis, a cooperative binding effect is observed. The binding site of DA to  $\alpha$ Syn is different from the Cu binding site, but together they affect the protein folding.<sup>72</sup> The combined effect of this binding is an enhanced propensity for  $\alpha$ Syn to oligomerize and EPR spectroscopy showed a higher ROS production in this constellation.<sup>73</sup> The oligomerization of  $\alpha$ Syn in the presence of Cu can also be enhanced by the DA metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is toxic and is produced during the metabolism of DA. In PC12 cells treated with Cu, the addition of L-DOPA, the chemical precursor of DA, results in the production of  $\alpha$ Syn dimers and DOPAL, giving a hint to why dopaminergic neurons are more affected in PD.<sup>74</sup>

The formation of  $\alpha$ Syn aggregates occurs also in presence of Cu alone. When recombinant  $\alpha$ Syn is incubated with Cu( $\pi$ ), the resulting aggregated forms present different morphologies. Cryo-EM images suggest that 60 hours of incubation give rise to annular, fibrillar and protofibrillar species. These species live for a relatively short time, and after 7 days of incubation only fibrils were present<sup>75</sup> (Fig. 2).

In summary, the interaction of Cu and  $\alpha$ Syn is characterized by specific and pH-dependent binding modes, it can be altered by pathogenic mutations of  $\alpha$ Syn and affect the aggregation propensity of the protein. This interaction can also promote noxious effects like increased ROS production that can be exacerbated by the presence of DA or its metabolites likely contributing to selective vulnerability of dopaminergic neurons.

#### $\alpha$ -Synuclein and manganese

In contrast to iron and copper, manganese (Mn) is much less abundant in the human body.<sup>45</sup> Nevertheless, it plays a prominent role in the development of the brain and muscles and can be found as a cofactor in several enzymes such as transferases, hydrolases and superoxide dismutase.<sup>34</sup>

The metabolism of Mn is largely overlapping Fe metabolism due to the similar red-ox behavior of these metals. In fact, in the CNS the main Mn importer is DMT1 while Fpn1 is responsible for its efflux.<sup>45</sup> It is noteworthy that also other proteins might be involved in Mn but not in Fe import such as ZIP proteins, ionotropic glutamate receptor Ca<sup>2+</sup> channels and TRPM7.<sup>45</sup> Exposure to elevated levels of Mn, *e.g.* as occupational exposure in welders, has been already known for a long time to result in manganism, a disorder which shares many phenomenological features with PD, such as cognitive decline, psychiatric alteration and movement abnormalities.<sup>34</sup> However, Mn can also be involved in the etiology of PD itself as could be noted in an epidemiological study of PD patients who were previously exposed to this metal.<sup>76</sup>

In biophysical studies using NMR, Mn(II) has shown a poor affinity for  $\alpha$ Syn: in the range of 1 mM. The binding site of Mn to  $\alpha$ Syn is in the C-terminus in the Asp-121, Asn-122, and Glu-123.<sup>51</sup> Mn failed in inducing  $\alpha$ Syn fibril formation in aggregation assays using Thioflavin T fluorescence, but in tyrosine fluorescence quenching assays Mn(II) can influence  $\alpha$ Syn folding<sup>18</sup> (Fig. 1).

As for many transition metals, one proposed mechanism for Mn toxicity is its ability to increase oxidative stress inside the cell. For example, organotypic brain slices from rats treated with Mn show bursts in ROS production and an increased amount of oligomeric  $\alpha$ Syn. The increase of oligomer presence was directly related to the concentration of the metal. Furthermore the oxidants occurrence (H<sub>2</sub>O<sub>2</sub>) seemed to exacerbate the process while the addition of anti-oxidant molecules (GSH) had the opposite effect.<sup>77</sup> These results are also supported by studies in non-human primates, in which injections of Mn trigger aggregate formation of  $\alpha$ Syn in the frontal cortex that appear similar to those found in MSA.<sup>78</sup>

Data from a human  $\alpha$ Syn transgenic mouse model lead to infer that increased oral Mn intake has an impact on DA turnover, although PD-like neurodegeneration was not observed, which could be due to length and dosage of Mn exposure and the experimental model chosen in this study.<sup>79</sup>

Exposure to Mn appears also to affect nuclear pathways, such as the increase of  $\alpha$ Syn expression<sup>81</sup> and apoptosis through the regulation of the NF- $\kappa$ B pathway.<sup>82</sup> The latter one is of particular interest since a murine model with the knock-out of this gene develops a pathology that shares features with human PD, including the presence of fibrillar  $\alpha$ Syn and the death of dopaminergic neurons thus supporting the link between Mn exposure and PD onset.<sup>83</sup> On the other hand, Mn treatment was exacerbating cellular toxicity induced by overexpression of  $\alpha$ Syn in neuroblastoma SK-N-MC cells, suggesting a synergistic action on cell survival/death pathways.<sup>80</sup>

To better understand the selective vulnerability of dopaminergic neurons, primary neuron cultures were exposed to Mn and studied by SXRF. The results suggested that dopaminergic neurons have a higher uptake of Mn compared to other neurons and that  $Mn^{2+}$  was more likely to enter the cells than  $Mn^{3+}$ . One of the reasons could be the increased expression of voltage-gated calcium channels in dopaminergic neurons, which also permit Mn influx<sup>84</sup> (Fig. 2).

It is remarkable that Mn is also a co-factor of PMR1, a Golgiresident  $Ca^{2+}/Mn^{2+}$  ATPase that has been shown to be involved in Ca-mediated toxicity induced by  $\alpha$ Syn in yeast, nematodes and fly models. In fact, upon deletion of PMR1, the susceptibility for Mn<sup>2+</sup> and  $\alpha$ Syn was markedly increased.<sup>85</sup> The functional link between  $\alpha$ Syn and Mn was recently further strengthened by the discovery that the overexpression of *ATP13A2 (PARK9*), another gene related to familial PD, was shown to rescue neuronal death induced by  $\alpha$ Syn. Interestingly, at least in yeast, ATP13A2 can also protect neurons from manganese toxicity, likely acting as a Mn transporter.<sup>86</sup>

Although less abundant than Fe or Cu, Mn is thus in no ways the smaller brother of the two. Next to a prominent role as environmental toxin, Mn seems to increase  $\alpha$ Syn toxicity, when proteins regulating its transport are impaired. On the other hand, the facilitation of  $\alpha$ Syn-oligomerisation and an increase in ROS production are common themes together with both, Fe and Cu.

# Possible therapeutic approaches targeting the interaction of $\alpha$ -synuclein and metals

Given the deleterious effects of elevated metal levels in the brain and the increased  $\alpha$ Syn toxicity upon metal interaction, three different therapeutic strategies can thus be proposed: blocking the interaction of transition metals with  $\alpha$ Syn, reducing the levels of unbound transition metals or trying to decrease the oxidative stress produced by metals.

The first approach implies the use of compounds that can stabilize the association of  $\alpha$ Syn with the membranes.<sup>87</sup> It is believed that  $\alpha$ Syn *in vivo* is in constant equilibrium of several different aggregation states and when this protein is firmly bound to the membrane it is less prone to aggregate and to interact with metals that are usually present in the cytosol.<sup>70,87</sup>

The second approach could be achieved *in vivo* through the administration of chelators.<sup>29</sup> These compounds, for example deferoxamine (DFO), are already used in clinic for other diseases (*e.g.* chronic iron overload due to erythrocyte transfusion) and there are some promising studies showing positive effects in cellular and animal models of PD. In neuronal-like BE2-M17 dopaminergic cells and in primary neuron cultures the addition of a DFO-based compound resulted in increased cell-survival when these cells were exposed to paraquat (an herbicide) together with the over-expression of A30P mutant of  $\alpha$ Syn.<sup>88</sup> DFO has recently proven to be also effective *in vivo*. In fact, in a rat model of  $\alpha$ Syn over-expression mediated by adeno-associated virus, the intranasal administration of DFO showed a positive effect on the behavior of the animals and slowed the aggregation rate of  $\alpha$ Syn.<sup>89</sup>

A similar approach was also undertaken with a different chelator, deferiprone (DFP). DFP is a chelator that is orally bioavailable and it can cross the blood brain barrier. The authors used the MPTP model to induce dopaminergic degeneration in mice and showed that chelation therapy could significantly reduce the labile iron pool and the biological damage by oxidative stress. In the same publication a pilot study on PD patients was described. The results were encouraging and showed a lower amount of Fe in the brain measured by MRI and an improvement of patients in the motor rating of the Unified Parkinson's Disease Rating Scale.<sup>90</sup>

Although further trials are required, these studies are highly encouraging and support the notion that the administration of a chelator that can regulate the metal homeostasis could be a beneficial approach to address the pathophysiology of PD.

Generation of ROS by metals is a general theme, which is not restricted to a certain element. Thus, a promising therapeutic approach could also be the use of anti-oxidants in this context. For example, in SH-SY5Y cells cultured with an excess of Cu(II) and overexpressing  $\alpha$ Syn, the presence of glutathione and ascorbic acid was shown to positively influence cell survival.<sup>71</sup> Dopaminergic neurons appear to be more susceptible to metalinduced red-ox stress in cell culture and application of GDNF and NT-4 could improve their viability.<sup>91</sup> The idea that oxidative stress induced by  $\alpha$ Syn can be relieved in cells using antioxidants is confirmed in a study in which dopaminergic neuron cultures are challenged in presence of aggregated  $\alpha$ Syn. In this model the supply of l-NAME (a powerful NOS scavenger) can significantly reduce oxidative stress in the cell somata and also in the processes.<sup>92</sup>

Except for cell cultures, the use of antioxidants has been also studied in rodent models of PD in which the disease is induced by oxidative stress, which is interesting in two ways: PD patients' brains have been shown to have higher levels of oxidative stress accompanied by low coenzyme Q10 levels. Coenzyme Q10 is the electron acceptor for complexes I and II and also a potent antioxidant.<sup>93</sup> Indeed, in a mouse MTPT model, animals that received a diet enriched in coenzyme Q10 and creatinine proved to have lower oxidation levels.<sup>94</sup> However, there are doubts about the translation of these findings in the clinics. While a smaller study involving 89 patients showed slight improvements in the Unified Parkinson's Disease Rating Scale after coenzyme Q10 treatment,<sup>95</sup> a larger phase III study with a cohort of 697 patients wasn't able to reproduce these significant positive effects.<sup>96</sup>

#### Conclusion and outlook

With the discovery of aSyn as highly relevant protein in PD etiopathology and the elucidation of  $\alpha$ Syn-metal interactions, transition metals moved again into the spotlight of neurodegeneration research. Although the interactions are now subject of intense research, many open questions remain: How are levels of transition metals regulated by a Syn? Do metals influence a Syn expression or its posttranslational modifications? How important is the compartmentalization of metals and aSyn? And finally, can we exploit the reduction of aSyn-metal interaction in a therapeutic manner? It is certain, that such therapies need to be initiated at the earliest possible time point in order to address pathological changes in a less advanced stage. Unfortunately, the diagnosis of PD today is based on purely clinical signs and more than half of neuronal dopaminergic projections are vanished at the time of first motor symptoms.<sup>97</sup> This may explain why promising animal trials have not yet been successfully translated into a human setting. Thus, early treatment must eventually be based on preclinical biomarkers indicating incipient disease at a non-symptomatic stage. Since aSyn and metals play such important roles in the pathophysiology, both may become also promising subjects to investigation in the search of preclinical biomarkers for PD.

#### Acknowledgements

We are indebted to the European Synchrotron Radiation Facility (ESRF), the SOLEIL synchrotron and the Advanced Photon Source (APS) at the Argonne National Laboratory for generously providing beamtime for our projects. EC and PL are supported by the Cluster of Excellence and DFG-Research Center Nanoscale Microscopy and Molecular Physiology of the Brain. PL is funded by the Else Kröner-Fresenius-Stiftung.

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