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# Unveiling the role of the pesticides paraquat and rotenone on α-synuclein fibrillation *in vitro*

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ABSTRACT

Epidemiological data have suggested that exposure to environmental toxins might be associated with the etiology of Parkinson's disease (PD). In this context, certain agrochemicals are able to induce Parkinsonism in different animal models *via* the inhibition of mitochondrial complex I, which leads to an increase in both oxidative stress and the death of nigrostriatal neurons. Additionally, *in vitro* experiments have indicated that pesticides are capable of accelerating the fibrillation of the presynaptic protein α-synuclein (aS) by binding directly to the protein. However, the molecular details of these interactions are poorly understood. In the present work we demonstrate that paraquat and rotenone, two agrochemicals that lead to a Parkinsonian phenotype *in vivo*, bind to aS *via* solvent effects rather than through specific interactions. In fact, these compounds produced no significant effects on aS fibrillation under physiological concentrations of NaCl. NMR data suggest that paraquat interacts with the C-terminal domain of the disordered aS monomer. This interaction was markedly reduced in the presence of NaCl, presumably due to the disruption of electrostatic interactions between the protein and paraquat. Interestingly, the effects produced by short-term incubation of paraquat with aS on the protein conformation resembled those produced by incubating the protein with NaCl alone. Taken together, our data indicate that the effects of these agrochemicals on PD cannot be explained via direct interactions with aS, reinforcing the idea that the role of these compounds in PD is limited to the inhibition of mitochondrial complex I and/or the up-regulation of aS.



## 1. Introduction

Parkinson's disease (PD) is the second most common age-related disorder after Alzheimer's disease and effects 1–2% of the population over 65 years of age (Langston, 2006). The clinical symptoms (resting tremor, bradykinesia, rigidity and postural dysfunction) are due to a loss of dopaminergic neurons in the substantia nigra pars compacta and a resultant dopamine (DA) deficiency in the striatum (Langston et al., 1983; Langston, 2006; Sulzer, 2007). Although the etiology of PD remains unknown, both the formation of potentially toxic intracellular α-synuclein (aS) deposits (called Lewy bodies and Lewy neurites) (Spillantini et al., 1998) and exposure to environmental toxins (Petrovitch et al., 2002; Kamel et al., 2007) are believed to correlative of neuropathogenesis.

Epidemiological studies have indicated an increased incidence of PD within populations that have been exposed to agrochemicals, including herbicides and insecticides (Priyadarshi et al., 2000). These compounds are also capable of inducing Parkinsonism in different animal models *via* the inhibition of mitochondrial complex I, which results in increased reactive oxygen species (ROS) production as well as selective degeneration of nigrostriatal neurons (Hartley et al., 1994). Furthermore, it has been reported that certain agrochemicals might affect aS aggregation directly or indirectly. For instance, exposing rodents to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), paraquat or rotenone leads to the up-regulation of aS (Manning-Bog et al., 2002; Chorfa et al., 2013). Moreover, a direct binding of aS to agrochemicals has been reported to increase the rate of aS fibrillation *in vitro*, thereby contributing to PD pathogenesis (Uversky et al., 2001a,b; Silva et al., 2013a,b). Among these compounds are herbicides (paraquat,

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diuron, trifularin), fungicides (dithiocarbamate), and botanical (rotenone) or synthetic (kepone, DDT) pesticides.

There exists compelling evidence indicating that oligomeric intermediates are the toxic entity generated during aS fibrillation (Follmer, 2014). In this context, compounds that stabilize aS oligomers rather than fibrils have been associated with the formation of toxic aS aggregates (Winner et al., 2011). If aS fibrillation represents a non-toxic pathway by shifting the equilibrium toward the consumption of potentially toxic oligomers, the fact that agrochemicals accelerate the aS fibrillation process argues against the hypothesis that direct interaction of these compounds with aS contributes to aS-mediated PD pathogenesis. To clarify this inconsistency, the molecular details of the interactions between aS and the agrochemicals paraquat and rotenone were investigated.

## 2. Experimental

*Fibrillation of aS*. The expression and purification of aS was performed as previously described by our group (Coelho-Cerqueira et al., 2013). The molar concentration of the aS monomer was determined by measuring absorbance at 276 nm using a molar extinction coefficient of 5600 $M^{-1}$ $cm^{-1}$. aS fibrillation was carried out by incubating 35 μM purified aS monomer in 10 mM sodium phosphate, pH 7.5, in the presence of varying concentrations of NaCl (0–200 mM). The aggregation assay was performed at 37 °C under agitation (350 rpm) using a Thermomixer Comfort equipment (Eppendorf, Hamburg, Germany) in a 96-well plate (Corning NBS 96-well white plate) which contained a single 3 mm glass (borosilicate) bead per well. Fibril formation was monitored by measuring Thioflavin-T (ThT) fluorescence using a single-time point dilution protocol. ThT fluorescence was evaluated in a Cary Eclypse Fluorimeter (Agilent Technologies, Santa Clara, USA) by excitation at 446 nm and collection of fluorescence emission at 485 nm. To investigate the effect of rotenone on aS fibrillation, 35 μM aS monomer was incubated in the presence of 100 μM rotenone, dissolved in 100% DMSO. An equivalent amount of pure DMSO (5% as final concentration) was used as a control. Concentrations of rotenone higher than 100 μM were not achievable in aqueous solution with 5% DMSO, a condition in which DMSO did not interfere significantly in the fibrillation process; however, paraquat is largely soluble in water and was evaluated in concentrations ranging from 0.1 to 1 mM. Both paraquat and rotenone were purchased from Sigma–Aldrich Co (St. Louis, USA).

*Nucleation-dependent fibrillation of aS*. For the fibrillation in seeding conditions, 35 μM aS monomer in 10 mM sodium phosphate, pH 7.5, in the presence or absence of 100 mM NaCl, was incubated with aS seeds (5%, w/w) at 37 °C without agitation. aS seeds were prepared by allowing 100 μM of aS monomer to incubate for 8–10 days at 37 °C under agitation (350 rpm) until mature fibrils were created. Fragmented fibrils were then produced *via* ultrasound sonication (60 min; 40 kHz). To induce fibrillation induced *via* sodium dodecyl sulfate (SDS), 50 μM aS monomer was incubated at 37 °C, without agitation, in the presence of 600 μM of SDS (12 molecules of SDS per aS monomer).

*NMR*. All NMR experiments were performed at 15 °C on a Bruker Avance III 800 MHz spectrometer (Bruker Biospin GmbH, Reinstetten, Germany) using an inverse-detection triple resonance *z*-gradient probe. The sample contained 200 μM purified $^{15}$N-aS in 10 mM sodium phosphate, pH 7.5, [10% $D_2O$ (v/v)], in the presence or absence of 100 mM NaCl. Agrochemicals were evaluated at concentrations of either 100 μM rotenone or 1 mM paraquat. aS plus 5% DMSO was used as a control for rotenone. Insoluble aggregates were removed *via* centrifugation. Spectra were acquired both before and after incubation of the samples at 37 °C with agitation (350 rmp) for 24 h. Phase-sensitive two-dimensional $^{1}$H–$^{15}$N Heteronuclear Single Quantum Coherence (HSQC) spectra were recorded using Echo-anti Echo gradient selection. TopSpin 3.1 was used for data acquisition. All spectra were processed with NMRPipe (Delaglio et al., 1995) and analyzed with NMRViewJ (Johnson and Blevins, 1994). Amide resonance assignments were made according to previously reported chemical shift assignments for the unfolded aS monomer (Rao et al., 2009) and chemical shift perturbations (CSP) were calculated as follows:

$$\mathrm{CSP} = \sqrt{\delta H^2 + \left(\frac{\delta N}{10}\right)^2}$$

where $\delta H$ and $\delta N$ refer to the chemical shift of $^{1}$H and $^{15}$N, respectively.

*Isothermal titration calorimetry* (*ITC*). ITC measurements were performed using a VP-ITC calorimeter from MicroCal, Llc (Northampton, USA). The titration of 50 μM aS with rotenone (100 μM) or paraquat (2 mM) involved 10 injections (10 × 25 μL or 10 × 10 μL, respectively) at 5-min intervals with constant stirring at 307 rpm. The temperature was set at 37 °C. The reference cell was filled with Milli-Q water. For the assays using rotenone, exactly the same concentration of DMSO (5%) was added to both aS solution (or buffer alone) and rotenone to minimize the thermal effects of dilution of DMSO in water. Any heat produced by the study compounds following dilution into buffer was subtracted from the raw data obtained with aS.

*TEM*. 10 μL aliquots of aS samples were deposited on a carbon-stabilized, parlodion-coated copper grid (300 Mesh). Samples were dried at room temperature. Images of Energy Filtered Transmission Electron Microscopy (EFTEM) were acquired at the Institute of Chemistry, University of Campinas (Unicamp), São Paulo, using a Carl Zeiss Libra 120 transmission electron microscope operated at 80 kV.

## 3. Results

*Rotenone and paraquat fail to accelerate aS fibrillation under physiological concentrations of NaCl*. aS fibrillation can be described as a nucleated polymerization process in which the unfolded monomer undergoes self-assembly to form oligomeric intermediates (nuclei) at the onset of aggregation, followed by monomer accretion and fibril growth. Thus, aS fibrillation kinetics display sigmoidal behavior with an initial lag-time phase (nucleation) followed by an exponential-growth phase (elongation) that evolves until a plateau is reached (formation of mature amyloid-fibrils). Fig. 1A and E indicates that both agrochemicals (100 μM rotenone or 500 μM paraquat) accelerate aS fibrillation in the presence of sodium phosphate, pH 7.5, which is in accordance with previous reports (Uversky et al., 2001a,b; Silva et al., 2013a,b). By analyzing the kinetic parameters extracted from the normalized curves displayed in Fig. 1C and G we verified that the duration of the lag-time and the $t_{½}$ (time required to reach 50% of maximum fibrillation) significantly decreased in the presence of the pesticides, while the elongation rates increased (Table 1). Our assays utilized a molar ratio of aS monomer:pesticide of ~1:3 (for rotenone) and ~1:15 (for paraquat). Rotenone did not produce significant effects on aS fibrillation at concentrations below 100 μM and higher concentrations were not evaluated due to the very low solubility of this compound in aqueous medium. However, paraquat is highly soluble in water and a concentration-dependent effect on aS fibrillation was observed in the absence of NaCl (Supplementary Fig. S1 A).

Next, we investigated the manner in which NaCl concentration effected the interaction of aS with paraquat or rotenone. The intention of this assay was not to reproduce the physiological

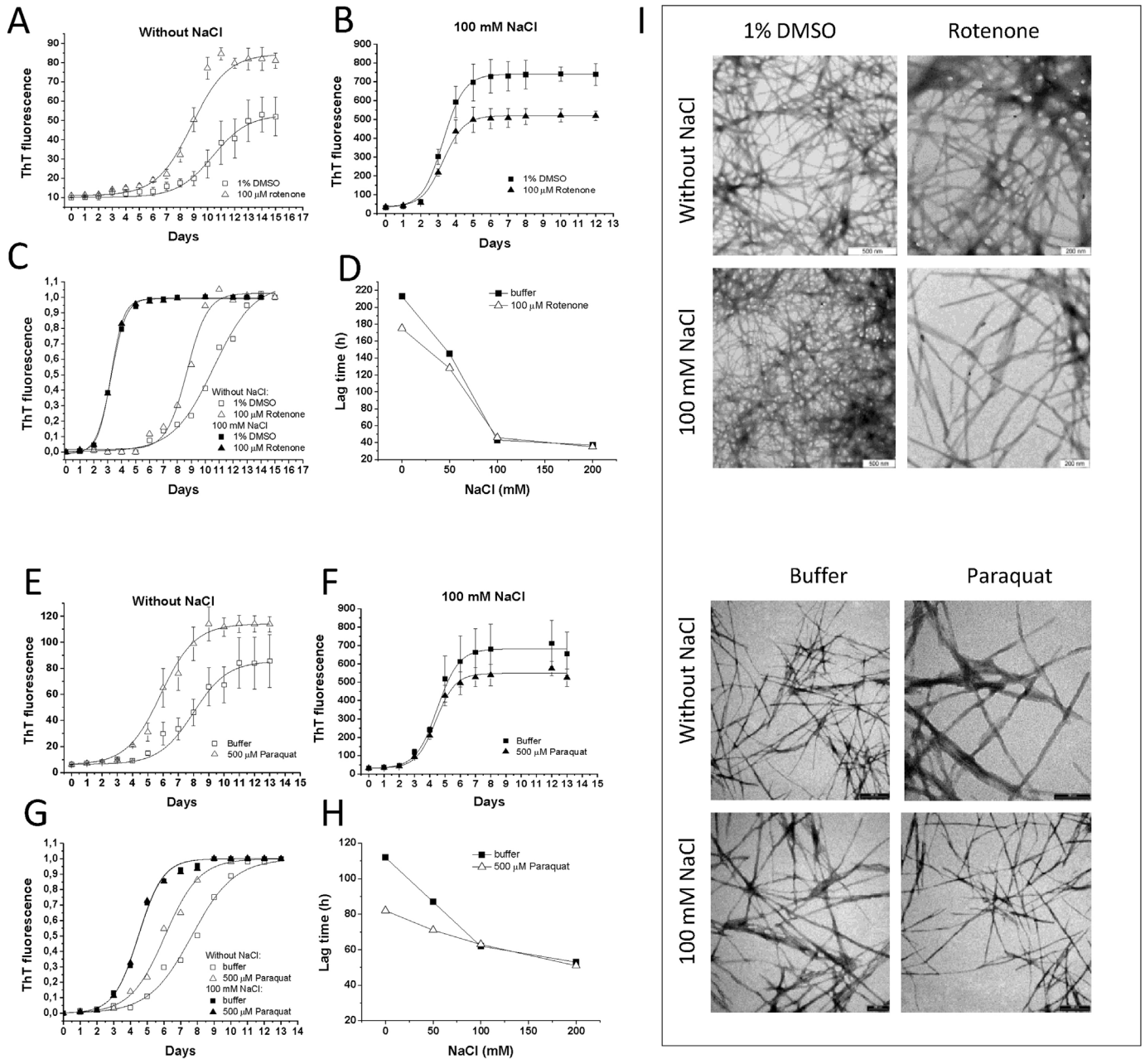


**Fig. 1.** Effects of the agrochemicals rotenone and paraquat on aS fibrillation. The effect of NaCl on the fibrillation of aS (35 μM in 10 mM sodium phosphate, pH 7.5) in the presence of either 100 μM rotenone (A–D) or 500 μM paraquat (E–H) was monitored by ThT fluorescence. (I) TEM images of the fibrils at the end of the incubation (12–15 days) generated under different conditions.

environment of aS in the presence of agrochemicals within a neuron but rather to investigate the influence of the ionic strength of the medium on aS-pesticide interactions and the consequences of such. Neither rotenone nor paraquat had any influence on the rate of aS fibrillation in the presence of 100–200 mM NaCl (Fig. 1B and F). In addition, no significant changes to the kinetic parameters of aS fibrillation were observed (Table 1), though a reduction in ThT fluorescence was noted at the end of aggregation in assays

**Table 1**
Effects of the agrochemicals paraquat and rotenone on the kinetic parameters of aS fibrillation in the presence or absence of NaCl.

| | 100 mM NaCl | | | Without NaCl | | |
|---|---|---|---|---|---|---|
| | $t_{1/2}$ (h) | Lag time (h) | Elongation rate ($h^{-1}$) | $t_{1/2}$ (h) | Lag time (h) | Elongation rate ($h^{-1}$) |
| aS (buffer) | 108 | 62 | 0.010 | 184 | 112 | 0.0057 |
| aS + 500 μM paraquat | 108 | 63 | 0.011 | 144 | 84 | 0.0077 |
| aS (5% DMSO) | 101 | 43 | 0.007 | 252 | 213 | 0.0065 |
| aS + 100 μM rotenone | 102 | 46 | 0.007 | 230 | 175 | 0.011 |

including the pesticides. The influence of varying concentrations of NaCl on the duration of lag-time, in the presence or the absence of the pesticides, is presented in Fig. 1D and H. We can clearly verify that the duration of the lag-time is nearly identical in the presence or the absence of the agrochemical when the aggregation assay was performed using NaCl concentrations higher than 100 mM. Even at a high paraquat concentration (1 mM), aS fibrillation kinetics were unaffected in the presence of 100 mM NaCl (Supplementary Fig. S1 A). Transmission electron microscopy (TEM) images indicated that neither paraquat nor rotenone caused any apparent alterations to fibril morphology (Fig. 1I). Additionally, these agrochemicals did not increase aS oligomer content even when in the presence of NaCl.

The concentrations of both paraquat and rotenone used in our assays are remarkable superior than those detected in animal models exposed to these pesticides. For instance, the subcutaneous administration of 20 mg/kg of paraquat in rats resulted in a serum and striatal extracellular concentration of ~8 and ~0.5 μM, respectively (Shimizu et al., 2001). Rotenone concentrations reached in animal models are in the nM range (Greenamyre et al., 2003). Considering that the physiological concentration of aS in brain has been estimated to be around 1 μM (Seo et al., 2002), the molar ratio of pesticides to aS monomer in our experiments might be considered hypothetically higher than that reached physiologically, which reinforces the idea of these pesticides likely do not have any effect aS fibrillation *via* a direct interaction.

*Paraquat and rotenone do not affect aS fibrillation in the presence of seeds or SDS*. The nucleation step of aS fibrillation may be abolished following the addition of preformed fibrils (seeds) to the aggregating mixture, which accelerates the process even in the absence of agitation (Yagi et al., 2005; Braga et al., 2011). Therefore, we prepared aS seeds by subjecting mature fibrils to ultrasound sonication and examined their effect on aS fibril formation. Fig. 2A–D shows that the addition of seeds to the aggregating mixture readily accelerates the formation of aS fibrils at 37 °C (no agitation). Instead of observing a lag phase, we measured an exponential increase in ThT fluorescence immediately following seed addition, independent of NaCl concentration. ThT fluorescence remained unchanged in the absence of seeds even during long-term incubation of the aggregating mixture at 37 °C (without agitation), indicating that neither paraquat nor rotenone produced measurable changes to aS fibrillation under seeding conditions.

It has been shown that aS fibrillation might be stimulated by the anionic detergent SDS though a mechanism unrelated to that obtained during seeding conditions (Giehm et al., 2010). In this alternative route, low concentrations of SDS (below the critical micellar concentration) accelerated fibril formation in a manner dependent on both the induction of an α-helical conformation within the protein and on the ability of SDS to form a scaffold for aS nucleation. In our assays, a molar ratio of 12 SDS molecules per monomer of aS was used to promote aS fibrillation, which was described by previous studies to induce aS fibrillation (Giehm et al., 2010). Fig. 2E–H demonstrates that the aS control (in buffer alone or buffer/2% DMSO) was capable of forming fibrils in the presence of SDS at 37 °C (without agitation), exhibiting an exponential increase in ThT fluorescence immediately following SDS addition. Furthermore, neither paraquat nor rotenone affected the process of SDS-induced aS fibrillation. These data corroborate the idea that the accelerative effect of these agrochemicals in the absence of NaCl is limited to the nucleation phase. In this case, neither paraquat nor rotenone produced an accelerative effect when nucleation was either abolished by seeds or SDS or shortened in duration by high NaCl concentrations.

*Probing the interaction between aS and paraquat/rotenone*. ITC was used to investigate the interaction of aS with paraquat or rotenone in the presence or absence of NaCl. Fig. 3A and B shows that rotenone interacts with aS by an endothermic process at 37 °C. A limited concentration of rotenone was used in this experiment due to its poor solubility in aqueous media. No observable saturation was visualized during the titration of aS with paraquat, which is indicative of a low-affinity interaction between the two compounds (Fig. 3C and D). Importantly, the presence of NaCl decreased the intensity of heat absorption following interaction of either of the agrochemicals with aS, suggesting that the binding of

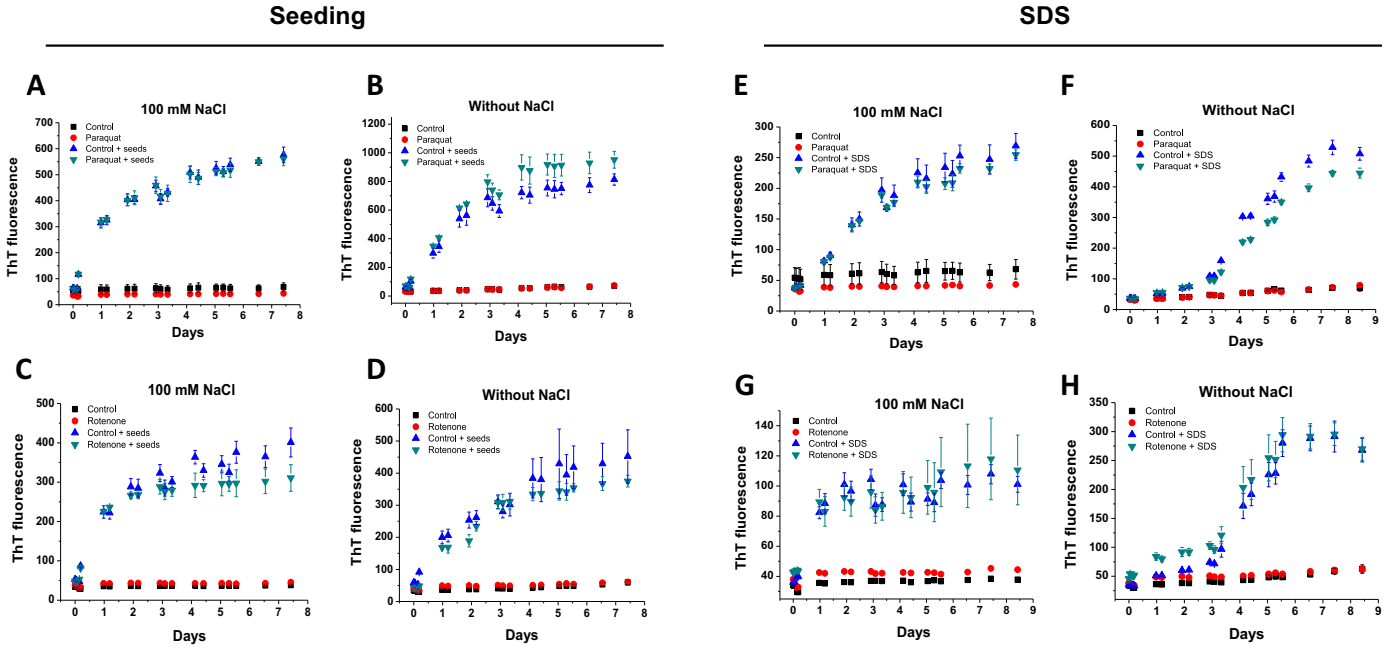


**Fig. 2.** Seeds and SDS-induced aS fibrillation in the presence of rotenone or paraquat. 35 μM aS monomer in 10 mM sodium phosphate, pH 7.5, in the presence (A and C) or absence of 100 mM NaCl (B and D), was incubated in the presence of seeds (5%, w/w) at 37 °C without agitation. For SDS-induced fibrillation (E-H), 50 μM aS monomer was incubated at 37 °C in the presence of 600 μM of SDS.

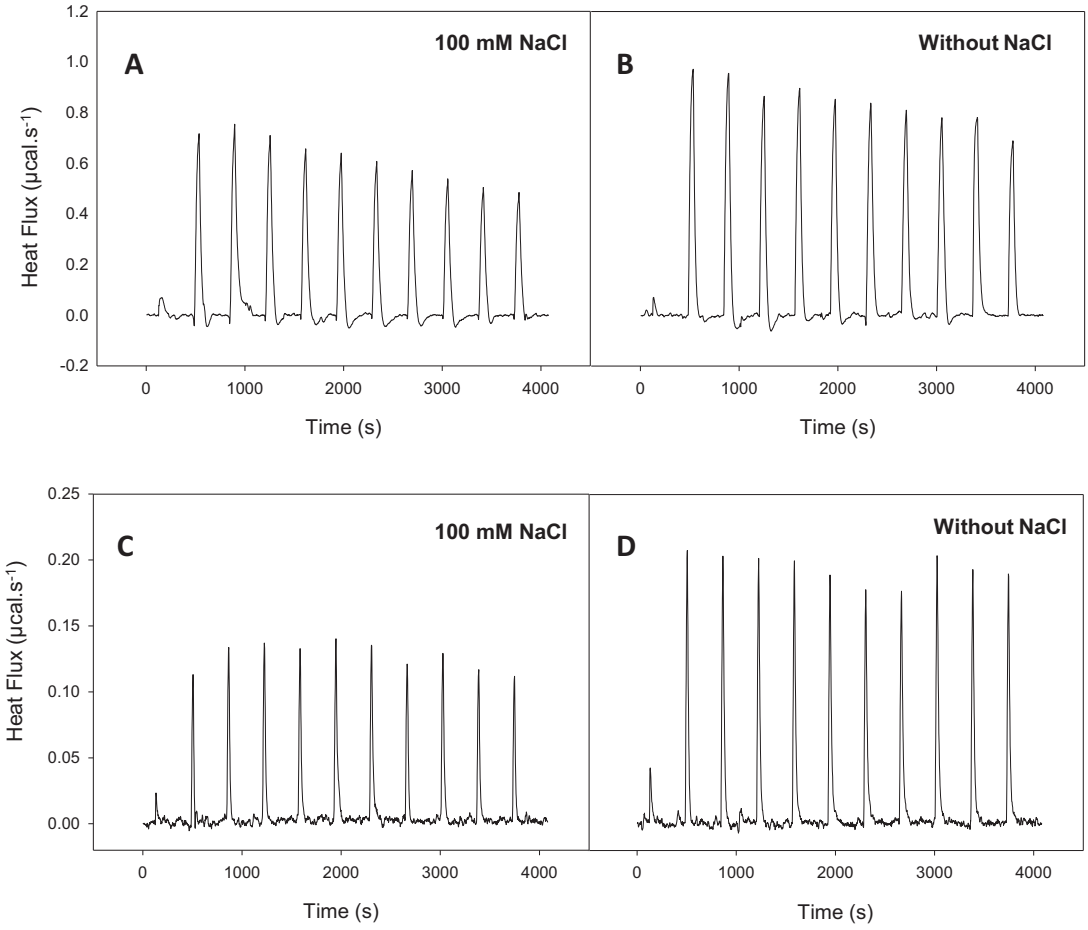


**Fig. 3.** ITC analysis of the binding of rotenone and paraquat to aS. Heat flux profiles associated with the injections of rotenone (100 μM) (A and B) or paraquat (2 mM) (C and D), in the presence or absence of 100 mM NaCl (as indicated) in the calorimetric cell at 37 °C. For the assays with rotenone, exactly the same concentration of DMSO (5%) was added to both the aS solution (or buffer alone) and rotenone to minimize the thermal effects produced by dilution of DMSO in water. The heat produced by compound dilution into buffer was subtracted from the raw data obtained with aS. For all experiments the aS concentration was 50 μM. The data are representative of three independent experiments.

these compounds to aS occurs primarily through electrostatic interaction.

To gain further information on the nature of the interaction between the aS monomer and paraquat or rotenone, the perturbations caused by the agrochemicals on the $^{1}H–^{15}N$-HSQC spectrum of the $^{15}N$-aS monomer were quantified by examining the intensity of CSP along the protein sequence. It is notable that there are three possibilities that may lead to perturbations in the signal of a certain residue in the aS $^{1}H–^{15}N$-HSQC spectrum, including: (1) direct binding of aS to the ligand, (2) changes to the microenvironment of the binding region, or (3) conformational changes produced by the ligand binding.

Rotenone did not produce any significant changes to the $^{1}H–^{15}N$-HSQC spectrum of aS, either in the presence or absence of NaCl (Supplementary Fig. S2). It is possible that the near absence of CSP observed within the $^{1}H–^{15}N$-HSQC spectrum of aS following incubation with ligand might be attributable to the low concentration of rotenone used in these NMR experiments. For rotenone, using a molar excess of ligand was not possible due to the low solubility of this compound in aqueous solution; in this case, a substoichiometric concentration of rotenone (100 μM rotenone to 200 μM aS) was used during NMR spectra acquisition. As a dynamic equilibrium exists between the bound and unbound states of aS to ligand, a molar excess of ligand is usually necessary to produce detectable perturbations in $^{1}H–^{15}N$-HSQC, especially when the binding constant is low. Paraquat's high aqueous solubility allowed us to utilize a 5-fold molar excess of ligand to protein during NMR analysis.

Fig. 4A shows the CSP in the $^{1}H–^{15}N$-HSQC spectrum of aS following introduction of 1 mM paraquat in the presence of 100 mM NaCl. Very slight perturbations were observed in the protein, most of which were located within the C-terminal region. Interestingly, CSP intensity was markedly increased when the spectra were acquired in the absence of NaCl (Fig. 4B). This suggests that NaCl is capable of shielding the interaction of paraquat with the C-terminal region of aS, which may explain the weak effect that paraquat has on aS fibrillation kinetics in the presence of salt.

*Paraquat prevents structural perturbations at the N-terminal of aS.* We next investigated the nature of the interaction between aS and paraquat following short-term incubation of the compounds at 37 °C with agitation. We first evaluated the conformational changes produced in aS following incubation with NaCl alone. In comparing the $^{1}H–^{15}N$-HSQC spectra obtained for aS before and after 24 h of NaCl incubation, we found that the highest CSP were produced within the N-terminal domain (residues 1–40) of aS when NaCl was absent from the assay buffer (Fig. 5A). No changes were observed at either the C-terminal or NAC domains. However, N-terminal perturbations were not observed when aS was assayed in the presence of 100 mM NaCl (Fig. 5B). Comparing the CSP

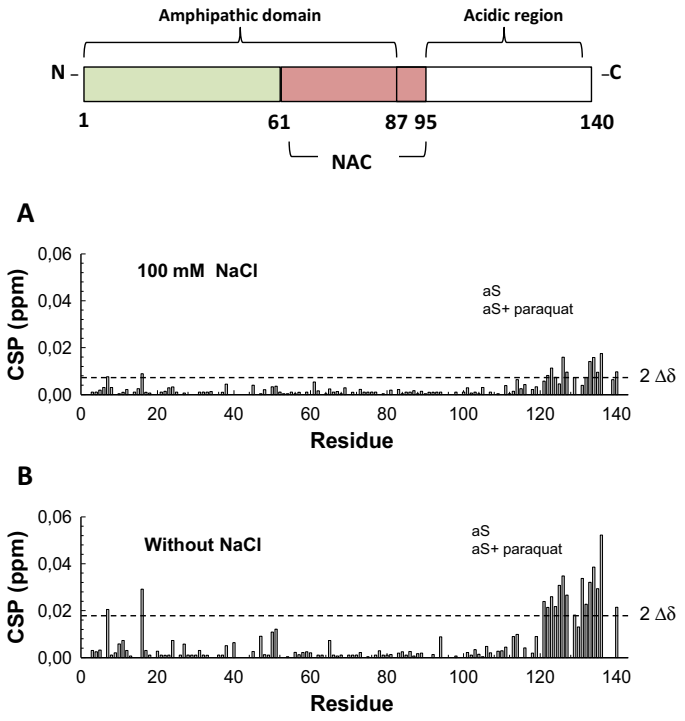


**Fig. 4.** CSP on $^{1}H–^{15}N$-HSQC spectrum of 15N-aS caused by 1 mM paraquat. The upper figure depicts a schematic representation of aS domains. The effect of paraquat on CSP was evaluated in the presence (A) or absence (B) of 100 mM NaCl. All experiments were performed at 15 °C using 200 μM $^{15}N$-aS in 10 mM sodium phosphate, pH 7.5, pH 7.5/10% $D_2O$.

intensity of aS when in the presence or absence of NaCl incubation confirms clearly that NaCl causes conformational changes to the N-terminal region of this protein (Fig. 5C).

To verify whether paraquat could prevent the above observed conformational changes to the N-terminus of aS, we compared the $^{1}H–^{15}N$-HSQC spectra of aS before and after a 24 h incubation with 1 mM paraquat. In contrast to the spectra obtained for aS alone, N-terminal CSP were not observed following the incubation of aS with paraquat (Fig. 5D). Moreover, the addition of 100 mM NaCl to the aS/paraquat assay buffer did lead to observable changes within the aS spectrum (Fig. 5E). These results indicate that the short-term incubation of aS with either NaCl or paraquat prevents structural perturbations to the N-terminal region of aS. In addition, these experiments allow us to probe whether paraquat exhibits a preference for interacting with either the aS disordered monomer (found at the initial time) or the aS conformer generated after a 24 h incubation. By comparing the results obtained in Fig. 5D with Fig. 4B, we were able to conclude that the binding of paraquat to aS did not change after short-term incubation, suggesting that paraquat preferentially interacts with disordered monomers of aS.

## 4. Discussion

A growing body of evidence suggests that exposure to environmental toxins is associated with neurodegeneration in sporadic PD (Petrovitch et al., 2002; Tanner et al., 2009). In this context, an association between PD and the exposure to pesticides such as paraquat and rotenone has been demonstrated through clinical and epidemiological research (Tanner et al., 2009, 2011). The underlying mechanisms behind paraquat and rotenone toxicity are directly or indirectly related to increased production

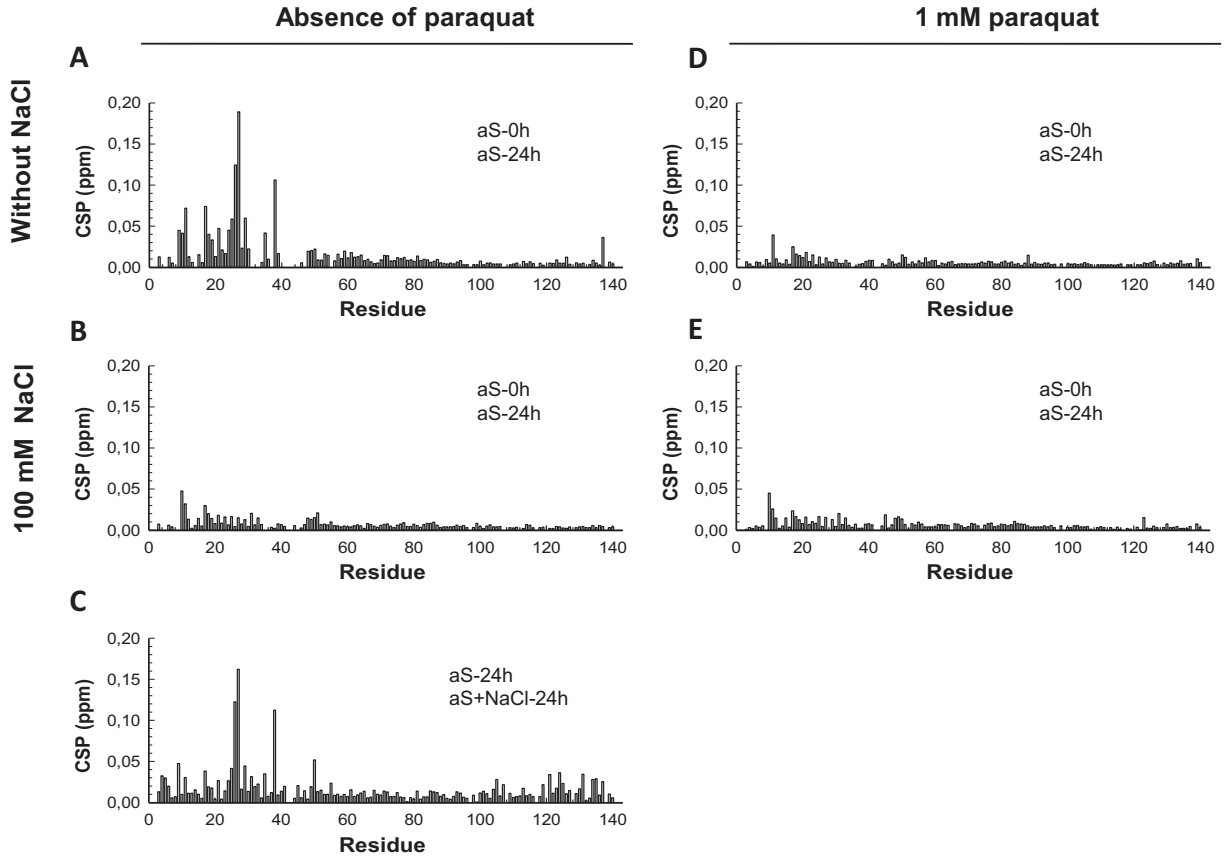


**Fig. 5.** Effect of short-term incubation at 37 °C on $^{15}N$-aS structure in the presence or absence of 1 mM paraquat. CSP in the $^{1}H–^{15}N$-HSQC spectrum of $^{15}N$-aS following 24 h of incubation at 37 °C was evaluated in the absence (A–C) or presence (D and E) of 1 mM paraquat. In (C), the effect of 100 mM NaCl on the $^{1}H–^{15}N$-HSQC spectrum of $^{15}N$-aS, after 24 h of incubation, was evaluated. Experiments were performed at 15 °C and using 200 μM $^{15}N$-aS in 10 mM sodium phosphate, pH 7.5, pH 7.5/10% $D_2O$.

of reactive oxygen species following exposure to these compounds. For paraquat, a redox cycle leading to the formation of paraquat monocation free radicals ($PQ^{\bullet+}$) leads to increased oxidative stress, notably due to the production of hydrogen peroxide and hydroxyl radicals which triggers injurious cellular effects (Dinis-Oliveira et al., 2006). An impairment of oxidative phosphorylation in mitochondria by inhibiting complex I of the electron-transport chain is associated to the toxic effects of rotenone (Hartley et al., 1994; Silva et al., 2013a,b).

In addition to increased cellular oxidative stress, another hypothesis to explain the association between pesticides and PD etiology suggests that these compounds can influence the formation of toxic aggregates of the presynaptic protein aS. Certain pesticides are capable of increasing the intracellular concentration of aS both *in vivo* and *in vitro*, which may be attributable to either alterations within the proteasomal pathway used for degradation of misfolded proteins, or to an increased synthesis of aS (Ulusoy and Di Monte, 2013). For example, paraquat exposure led to both up-regulation of aS within SN neurons in mice, and the formation of Thioflavin-S positive aggregates of the protein (Manning-Bog et al., 2002). It is notable that paraquat shares structural features with the neurotoxin $MPP^+$ (1-methyl-4-phenylpyridinium) (Fig. 6), which also triggers Parkinsonism in humans and non-human primates and promotes the up-regulation of aS in the SH-SY5Y cell line (Gómez-Santos et al., 2002).

Alternatively, some studies have demonstrated that pesticides directly bind to aS resulting in an increased aS fibrillation rate *in vitro* (Uversky et al., 2001a,b; Silva et al., 2013a,b). However, the existence of a direct interaction between aS and pesticides that leads to an acceleration of the formation of aS fibrils has not been identified by any *in vivo* experiment. Increased aS levels following exposure to rotenone, paraquat, and maneb were measured in two human cell lines (SH-SY5Y neuroblastoma and SK-MEL-2 melanoma) modified to endogenously express aS (Chorfa et al., 2013). In these experiments, only monomers of aS were detected (as opposed to high molecular weight aggregates). Interestingly, when mice were exposed to paraquat, the overexpression of aS did not lead to an increase in cell death, and paraquat also did not worsen the behavioral deficits caused by the overexpression of human aS (Fernagut et al., 2007). Additionally, it has been suggested that the intermediates of the fibrillation process (oligomers), instead of the fibrils themselves, are the toxic species generated during aS aggregation (Lashuel et al., 2002; Luth et al., 2014; Rockenstein et al., 2014). For example, aS mutations that stabilized an oligomeric state of the protein led to a more severe loss of dopaminergic neurons within the substantia nigra of rats than did mutations that stabilized fibrils (Winner et al., 2011). Taken together, these data cast doubts on whether the direct binding of aS to either paraquat or rotenone as demonstrated by a series of *in vitro* experiments would even be relevant under physiological conditions.

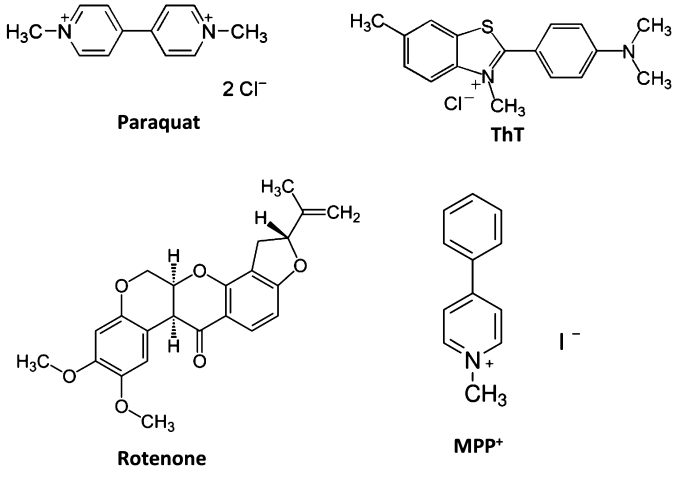


**Fig. 6.** Chemical structure of the accelerators of aS fibrillation *in vitro*: paraquat, rotenone, $MPP^+$ and ThT.

In biological systems, proteins are solvated in an aqueous environment containing salt ions and numerous other solutes, which might influence their interactions with specific ligands through diverse mechanisms (electrostatic screening, hydrophobic or Hofmeister effects, *etc.*) (Munishkina et al., 2004). In this context, we investigated how NaCl concentration effects the interaction of aS with paraquat or rotenone. Our findings are contrary to the current hypothesis that these agrochemicals stimulate the aS fibrillation. In fact, we demonstrate here that neither paraquat nor rotenone are capable of increasing the rate of aS fibrillation in the presence of physiological concentrations of NaCl. Thus, any profibrillogenic effects of these compounds on aS are restricted to conditions with low ionic strength, where nonspecific interactions are favored. Furthermore, these compounds produced no effect on aS fibrillation rates under conditions in which fibrillation can be stimulated by additives that reduce lag-time (such as seeds or SDS). The influence of media composition on aS-pesticide binding has been neglected in previous studies, and this lack of appropriate control may explain discrepancies amongst findings produced in such work.

The presence of NaCl also produced a decrease in the intensity of heat absorption following interaction of either agrochemical with aS, as verified by ITC experiments. It is noteworthy that the ITC binding profiles observed herein might be associated with alternative thermodynamic processes that, when summed up, result in an entropically driven process. Thus, increasing NaCl concentrations might affect these measurements either positively or negatively, with only a net effect being detected. Therefore, the ITC data obtained in our studies can only indicate that NaCl affects the energy of interaction between aS and paraquat or rotenone but does not provide information on the mechanisms driving this interaction.

aS is comprised of three main domains: an N-terminal domain (residues 1–61) containing four imperfect KTKEGV repeats with a propensity to form α-helical structures (Eliezer et al., 2001; Chandra et al., 2003); a highly hydrophobic internal portion (residues 61–95) [non-amyloid component (NAC)] that plays an essential role in fibrillation; and an unstructured C-terminal domain (residues 95–140) (Hoyer et al., 2004; Hong et al., 2011). CSP analysis in the $^{1}H–^{15}N$ HSQC spectra of aS indicates that the most significant alterations caused by the exposure of aS to paraquat were found within the C-terminal region (Fig. 4B). Importantly, the intensity of these CSP nearly disappeared in the presence of NaCl, which may explain the lack of change produced by paraquat on fibrillation kinetics in the presence of salt. Note that the values of CSP observed here (0.02–0.06 ppm) are either within range or superior to those found for other compounds that bind to aS and influence protein fibrillation, such as $Ca^{2+}$ ions (Zhang et al., 2014), ThT (Coelho-Cerqueira et al., 2014), multiple forms of vitamin K (which are described to bind to the N-terminal of the aS monomer and inhibit protein fibrillation (Silva et al., 2013a,b), and even UV-irradiated aS (Carmo-Gonçalves et al., 2014). Similar to paraquat, ThT and $Ca^{2+}$ ions accelerate aS fibrillation *in vitro* in the absence of NaCl, with the C-terminus being the main region of aS that is affected by the presence of these ligands. Curiously, paraquat and ThT molecules display certain structural similarities (Fig. 6). Therefore, the intensity of CSP observed in our study is in accordance with previous reports for other ligands of aS. The location of these perturbations within the aS sequence additionally showed high reproducibility amongst varying spectrum acquisitions (data not shown) despite that the intensity of CSP values may

display slight alterations in response to small differences in temperature, pH, ionic strength and concentration. Importantly, in all cases the values of CSP were higher in the absence of NaCl.

Considering that aS is highly charged at physiological pH with the majority of negatively charged residues being localized within the C-terminal region of the protein, the positively charged paraquat could stimulate aS fibrillation by neutralizing the intermolecular repulsion caused by the negatively charged C-terminus. Neutralization of the Coulombic charge–charge repulsion between protein monomers, which favor fibrillation by increasing local concentration, is also thought to be the dominant effect associated with the conformational changes and profibrillogenic action promoted by NaCl, certain metal ions (Uversky et al., 2001b) and polycations (Goers et al., 2003). On the other hand, long-range intra-molecular electrostatic interactions of the C-terminal region with either NAC or the N-terminal domain of aS stabilize the disordered monomer and any perturbation of them might stimulate or inhibit aS fibrillation (Hoyer et al., 2004; Hong et al., 2011). In this context, we can speculate that the binding of paraquat to the C-terminal of aS is potentially destabilizing to its interaction with other regions of aS, such as the N-terminal or NAC domain, thereby facilitating the conversion of the disordered polypeptide into a β-sheet conformation.

aS aggregation is associated with both hydrophobic and electrostatic interactions between protein residues, as well as with changes in protein hydration (Munishkina et al., 2004). In this context, the influence of salt on protein conformation and aggregation might be attributable to ions binding directly to either protein residues or water molecules, potentially perturbing water–water and protein–water interactions and changing the hydrophobic interactions of the protein. Far-UV circular dichroism experiments have indicated that NaCl induces a completely reversible increase in the content of ordered secondary structure within aS, leading to a partially folded fibrillogenic aS intermediate (Goers et al., 2003). Similarly to NaCl, pesticides and certain metal ion are also thought to favor this partially folded conformation and accelerate aS fibrillation (Silva et al., 2013a,b). Thus, when the aS fibrillation assay is performed in the presence of NaCl, the salt can affect aS fibrillation by both stabilizing a partially folded fibrillogenic monomer (*via* intramolecular interactions) and by neutralizing the Coulombic repulsion between the protein monomers (*via* intermolecular interactions) which together might mask the accelerative effect of paraquat or rotenone on aS aggregation.

In conclusion, our data indicate that paraquat and rotenone appear to interact with aS *via* solvent effects rather than specific interactions, as their effects on aS fibrillation kinetics depend on NaCl concentration. For example, a slight increase in NaCl concentration is sufficient to abolish any accelerative action of paraquat and rotenone on aS fibrillation *in vitro*. Based on these findings, we propose a revision of the putative profibrillogenic action of rotenone and paraquat on aS, and suggest that the primary effect of these compounds on PD is more likely associated with an increase in oxidative stress and/or the up-regulation of aS *versus* the acceleration of aS fibrillation through direct protein–pesticide interactions.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.11.006.

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