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Pramipexole Reduces Phosphorylation of α-Synuclein at Serine-129

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Abstract α-Synuclein is a central component of the pathogenesis of Parkinson's disease (PD). Phosphorylation at serine-129 represents an important post-translational modification and constitutes the major form of the protein in Lewy bodies. Several kinases have been implicated in the phosphorylation of α-synuclein. The targeting of kinase pathways as a potential to influence the pathogenesis of PD is an important focus of attention, given that mutations of specific kinases (LRRK2 and PINK1) are causes of familial PD. Pramipexole (PPX) is a dopamine agonist developed for the symptomatic relief of PD. Several in vitro and in vivo laboratory studies have demonstrated that PPX exerts neuroprotective properties in model systems of relevance to PD. The present study demonstrates that PPX inhibits the phosphorylation of α-synuclein and that this is independent of dopamine receptor activation. PPX blocks the increase in phosphorylated α-synuclein induced by inhibition of the ubiquitin proteasomal system. The phosphorylation of α-synuclein occurs in part at least through casein kinase 2, and PPX in turn reduces the phosphorylation of this enzyme, thereby inhibiting its activity. Thus, PPX decreases the phosphorylation of α-synuclein, and this mechanism may contribute to its protective properties in PD models.

Keywords Parkinson's disease · Neurodegeneration · Pramipexole · α-Synuclein · Phosphorylation · Neuroprotection · Dopamine agonists

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised in its early stages by the motor features of bradykinesia, rigidity and tremor, which in turn are related predominantly to dopamine cell loss in the substantia nigra pars compacta. This pathology, together with degeneration of other neurotransmitter systems, is also associated with non-motor symptoms that evolve with disease progression (Chaudhuri et al. 2006; Fox et al. 2008; Chaudhuri and Schapira 2009). Although dopamine replacement therapy can improve quality of life and life expectancy, a major unmet need in PD is the ability to slow or stop the progression of neurodegeneration (Olanow et al. 2008; Schapira et al. 2009).

The pathogenesis of PD is thought to involve a number of intersecting pathways that include mitochondrial dysfunction, oxidative stress, lysosomal abnormalities and protein aggregation (Schapira 2006). A substantial number of compounds have been proposed to have an effect of slowing PD degeneration by interfering with these pathways, based mainly on the results of laboratory studies (Schapira 2009a, b). Dopamine agonists and monoamine oxidase B inhibitors have demonstrated neuroprotective properties in a variety of in vitro and in vivo models of PD and are thought to exhibit antioxidant and anti-apoptotic properties (for reviews, see Schapira et al. 2009; Schapira 2009a).

The deposition of α-synuclein as the major constituent of Lewy bodies and the discovery that mutations and multiplications of the α-synuclein gene are causes of familial PD have focussed attention on this protein in the pathogenesis of PD (Fuchs et al. 2008; Ross et al. 2008). It is notable that the predominant form of α-synuclein in Lewy bodies is phosphorylated at its serine-129 residue (Fujiwara et al. 2002; Anderson et al. 2006), and this phosphorylation is increased in A53T α-synuclein expression (Wakamatsu et al. 2007). The kinase pathways that perform this phosphorylation have not

yet been fully identified and may involve multiple sequential stages. The phosphorylated form of α-synuclein can be toxic to dopaminergic cells and this is increased by oxidative stress and proteasomal inhibition (Chau et al. 2009). Post-translational modification in the form of phosphorylation appears to be of significance to a number of pathways involved in dopaminergic cell death (Dagda et al. 2009; Obeso et al. 2010; Schapira and Tolosa 2010; Sha et al. 2010).

Several studies have demonstrated that mitochondrial function may be influenced by α-synuclein (Devi et al. 2010; Nakamura et al. 2011), although the role that serine-129 phosphorylation may play in this has not been elucidated. The D2/D3 dopamine agonist pramipexole (PPX) has demonstrated that it is capable of reducing cell death in toxin models of dopamine loss in vitro and in vivo and that this may in part be through an action on mitochondria possibly at the level of the permeability transition pore (Schapira 2008). Our studies on PPX and its potential for neuroprotection led us to investigate the relationship between this compound and α-synuclein phosphorylation. We hypothesised that PPX may reduce phosphorylation at the serine-129 residue site of α-synuclein and that this contributes to the drug's protective role in PD models.

Materials and Methods

Cell Culture

A human dopaminergic cell line SH-SY5Y, stably overexpressing human wild-type α-synuclein tagged with haemagglutinin (HA) at its C terminus was cultured as previously described (Chau et al. 2009). Cell culture reagents were supplied by Life Technologies (Paisley, UK).

Treatments and Samples Collection

Cells were plated at subconfluent density, to which PPX and its enantiomer SND919CL2x (SND) (Boehringer Ingelheim GmbH, Ingelheim, Germany), dopamine receptors antagonist clozapine (Tocris, R&D Systems, Abingdon, UK) and epoxomicin (Calbiochem, San Diego CA, USA) were added. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was obtained from Enzo Life Sciences (Exeter, UK), whereas 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) was obtained from Sigma-Aldrich (Dorset, UK). Where kinase activities were measured, okadaic acid (OKA; Calbiochem, San Diego CA, USA) was added to 1 μg/μl 30 min before cells were harvested. Upon collecting the samples, cells were washed with phosphate-buffered saline (PBS) twice and lysed in situ in a buffer containing 1 % Triton X-100, 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and 50 μM NaF. Cell lysates were collected and spun at 21,000×*g* at 4 °C for 10 min, and soluble materials were retained for Western blot analysis. Bafilomycin A1 was obtained from Sigma-Aldrich (Dorset, UK).

Western Blot Analysis and Densitometry

Equal amounts of protein (as determined by bicinchoninic acid [BCA] assays; Pierce, Rockford, IL, USA) from the soluble materials were resolved under reducing conditions in NuPAGE 4–12 % polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), using the MES buffer, and transferred onto Immobilon PVDF membrane (Millipore, Billerica, MA, USA). Antibodies detecting HA epitope (Covance, Princeton, NJ, USA), phospho-serine-129 (P-S129) of α-synuclein (Wako, Neuss, Germany), α-synuclein (clone LB509; Invitrogen, Carlsbad, CA, USA), β-actin (Abcam, Cambridge, UK), phospho-serine-209 (P-S209), pan-casein kinase 2-β (Abcam, Cambridge, UK) and ubiquitin (Abcam, Cambridge, UK) were used, and the secondary antibodies detecting mouse and rabbit IgG were supplied by Dako (Glostrup, Denmark). ECL reagent (GE Healthcare, Bucks, UK) was used to develop the immunostained blots, and signals were detected by ECL films (GE Healthcare, Bucks, UK). Images on X-ray films were scanned with a trans-illuminating light source and densities measured using the DigiDoc gel analysis software (Alpha Innotech, East Sussex, UK).

Measurements on Lactate Dehydrogenase Release

Cell death was quantified by lactate dehydrogenase (LDH) release as described in Chau et al. (2009) using the kit manufactured by Promega (Southampton, UK),

Quantification of α-Synuclein Oligomerisation

Total level of α-synuclein was measured using the enzyme-linked immunosorbent assay (ELISA) kit supplied by Life Technologies (Paisley, UK). α-Synuclein oligomers was quantified using the same ELISA kit, with the detection antibody replaced with the oligomer antibody clone A11 (Life Technologies, Paisley, UK). Data were expressed as immunoactivity of A11 relative to the antibody detecting the total level of α-synuclein.

Biochemical Kinase Profiling

In vitro inhibition of a kinase library by 10 μM PPX was measured commercially by SelectScreen biochemical kinase profiling (Life Technologies, Paisley, UK).

Proteasomal Activity Measurements

Cells collected, after being washed with PBS twice, were frozen and thawed three times in 10 mM Tris–HCl pH 7.5.

Cell lysates were loaded onto a black 96-well plate and mixed with the respective fluorogenic substrate for PGP, chymotrypsin and trypsin-like activities (Calbiochem, San Diego CA, USA) at 80 μM final concentration. V_{max} was measured in a fluorescent plate reader with the chamber temperature set to 32 °C for 20 min at excitation and emission of 380 and 460 nm, respectively. V_{max} was corrected by protein concentration as determined by the BCA assays.

Statistical Analysis

Paired *t* tests were conducted where two groups were compared, and one-way analysis of variance (ANOVA) followed by Dunnett's post test was used for multiple comparisons to the control group. In both cases, $p<0.05$ was deemed significant.

Results

Pramipexole Decreased the Constitutive Level and Formation Rate of α-Synuclein Phosphorylation at Serine-129

To establish marker measurements, levels of P-S129 and the total ectopic level of α-synuclein were measured by Western blot analysis on 1 % Triton cell lysates, using the antibodies specifically detecting P-S129 (as demonstrated in Fig. 1 in Chau et al. 2009 and Fig. S1) and HA, respectively. In our hands, the endogenous level of α-synuclein in SH-SY5Y cells is undetectable (Chau et al. 2009 and Fig. S1). We found no evidence that α-synuclein became Triton-insoluble under hyper-phosphorylation condition (supported by data in Fig. 2 in Chau et al. 2009 and Fig. S2), justifying the examination of only the Triton-soluble cell lysates in our system.

Figure 1a is a graphical presentation of the data to illustrate the time-dependent and dose-dependent relationship of PPX treatment in decreasing the constitutive P-S129 α-synuclein level. The data have been normalised to the control (represented as 100 %; mean, 100.5±11.2 %). The reduction in P-S129 levels became significant at 48 h for 10 or 100 μM PPX and 100 μM for its dopamine receptor inactive enantiomer SND, with further time-dependent decreases to 72 h for 100 μM PPX. These data indicate that the effect on P-S129 is independent of the dopamine receptors. Further demonstration of PPX acting independently of the dopamine receptors came from the use of the dopamine receptor antagonist clozapine co-incubated with PPX (Fig. S3). Clozapine had no effect on P-S129 α-synuclein. Figure 1b shows the typical Western blot images of the cells treated with a range of PPX concentrations or its enantiomer SND for a period up to 72 h, immunostained with the antibody detecting P-S129 and total levels of α-synuclein and β-actin.

Treatment with the phosphatase inhibitor OKA over 30 min led to an accumulation of P-S129 α-synuclein that allowed measurement of the rate of P-S129 formation and the activity of the kinases responsible for phosphorylation (Chau et al. 2009). Shown in Fig. 1c is a graphical representation of Western blot images (normalised to the control as 100 %; mean, 96.2±5.6 %), and Fig. 1d is a typical image of the cells treated with 100 μM PPX or SND for a period up to 72 h, followed by OKA. Our data demonstrate a time-dependent decrease in the rate of P-S129 α-synuclein formation with PPX or SND treatment, becoming statistically significant at 72 h. In our system, the kinases contributing to P-S129 α-synuclein appear to be inhibited by PPX in a dopamine receptor-independent manner.

Pramipexole Reduced the Proteasome Inhibition-Induced Level and Formation Rate of α-Synuclein Phosphorylation at Serine-129

In view of proteasomal inhibition as a model of PD (Imamura et al. 2008; Li et al. 2010), we studied this effect with epoxomicin on the levels of P-S129. Epoxomicin (at 14 nM for 24 h) significantly induced the constitutive level of P-S129 α-synuclein as well as the rate of its phosphorylation (Fig. 2a–c). Both PPX and its enantiomer SND pre-incubated for 48 h reduced the rate and levels of P-S129, increased upon epoxomicin treatment, in a dose-dependent manner that was significant at 100 μM. These data suggest that the kinases responsible for P-S129 α-synuclein were upregulated by proteasomal inhibition and negatively controlled by PPX independently of its dopamine receptor property.

Influence of PPX on α-Synuclein Phosphorylation at Serine-129 Induced by Proteasome Inhibition Does Not Appear to be Related to Cell Viability and α-Synuclein Oligomerisation

We determined whether PPX prevents cell loss upon epoxomicin treatment. The 24-h treatment with epoxomicin at 14 nM caused no noticeable death of SH-SY5Y cells (data not shown) despite the typical fourfold increase in P-S129 α-synuclein. Epoxomicin treatment at 14 nM for 72 h produced a 49±2 % LDH release, but no protection is seen upon co-incubation with PPX (Fig. S4A) despite the typical reduction of P-S129 α-synuclein. Similar observations were made by Imamura et al. (2008) from using another proteasome inhibitor lactacystin.

We also determined whether increased P-S129 α-synuclein mediated by proteasome inhibition is associated with oligomerisation of α-synuclein in vivo. Following treatment with epoxomicin or bafilomycin (known to cause α-synuclein accumulation and oligomerisation), we

captured all α-synuclein, followed by detecting either the total level of α-synuclein or the α-synuclein oligomers by the A11 antibody using an ELISA approach. Figure S4B shows that, unlike bafilomycin treatment, epoxomicin treatment for 24 h produced a small signal of α-synuclein oligomerisation, and it did not seem to be influenced by PPX co-incubation. Our observation is in contrast to that seen in vitro by Kakimura et al. (2001), in which PPX produced an inhibitory effect on α-synuclein aggregation induced by H_2O_2.

α-Synuclein Phosphorylation at Serine-129 was Dependent on Casein Kinase 2

A number of kinases have been identified to contribute to the formation of P-S129 α-synuclein (Waxman and Giasson 2008; Mbefo et al. 2010; Braithwaite et al. 2012). Previously, we demonstrated that casein kinase 2 (CK2) was responsible for half of the kinase activity phosphorylating α-synuclein at serine-129 in our model (Chau et al. 2009). In this study, a more specific CK2 inhibitor, TBB (used at 10 μM for 30 min), was found to significantly reduce the constitutive and epoxomicin-induced levels and rates of generation of P-S129 α-synuclein (Fig. 3a–c). These data provide additional support for the predominant position of CK2 as the kinase in both cases of the constitutive and induced P-S129 α-synuclein.

Fig. 2 PPX-induced D2/D3-independent reduction of the level and rate of α-synuclein phosphorylation at serine-129 (P-S129) by proteasome inhibition in SH-SY5Y cells. Epoxomicin (14 nM for 24 h) significantly (by *t* test) induced the constitutive level (**a**) and rate (**b**) of P-S129, whereas PPX or SND (when pre-incubated for 48 h and co-incubated with Epox) decreased the induced level and rate of P-S129, in a dose-dependent manner, reaching significance (by ANOVA followed by Dunnett and by *t* test) at 100 μM. Shown are the mean±SEM signals of P-S129 corrected by HA of six experiments. **c** A typical Western blot image reflecting data shown in **a** and **b**

Pramipexole Decreases the Abundance of P-S209 of Casein Kinase 2-β

As the first step to delineate how PPX controls the kinase responsible for P-S129 α-synuclein, we found that both PPX and the enantiomer (at 100 μM after 48 h) significantly reduced the phosphorylation of the β-subunits of CK2 at serine-209 (Fig. 4a, b), whereas the endogenous expression of CK2 was unaffected. These data have shed light to the potential influence of PPX to an upstream signal transduction pathway of CK2 in phosphorylating α-synuclein at serine-129.

Pramipexole Does Not Function as an Inhibitor to CK2 In Vitro

Next, we asked the question whether PPX (at 10 μM) functions as a kinase inhibitor in vitro. On a panel of 27 candidate

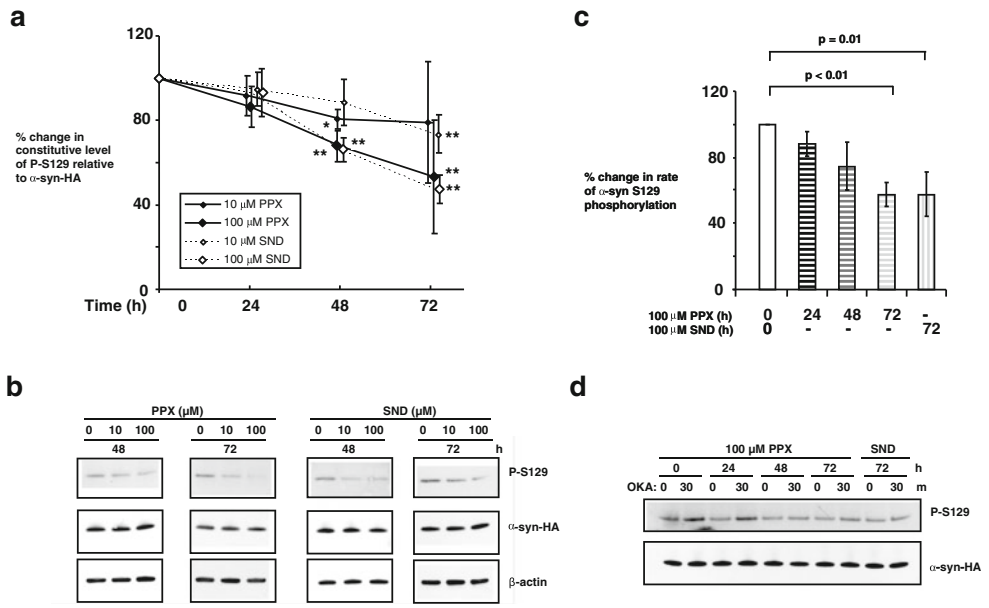
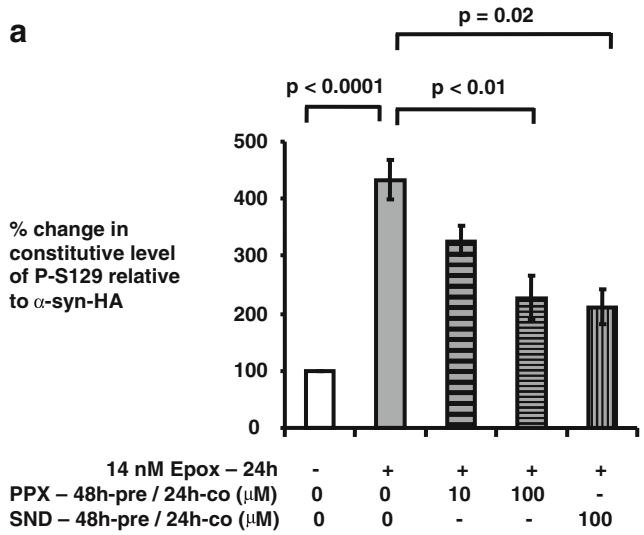
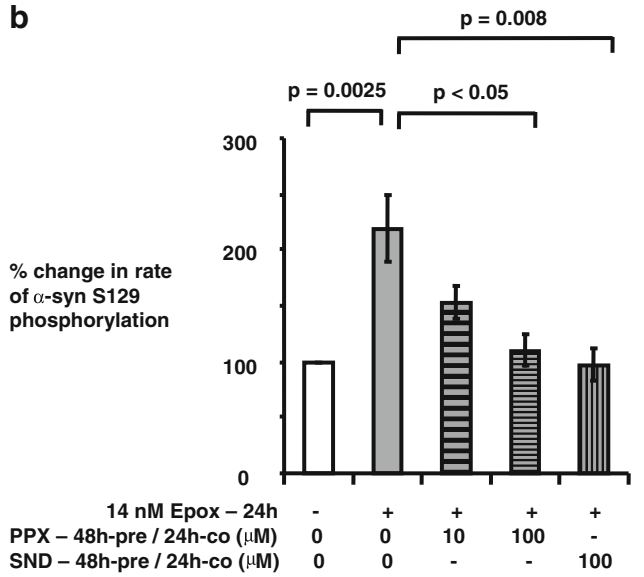
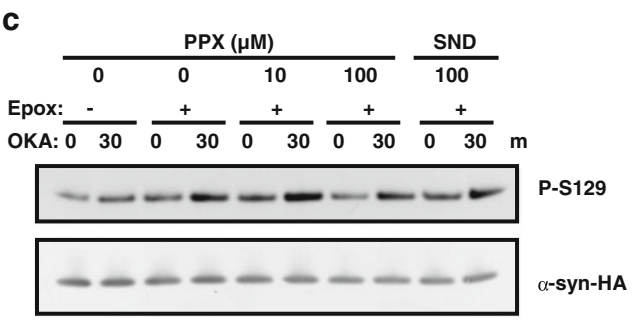
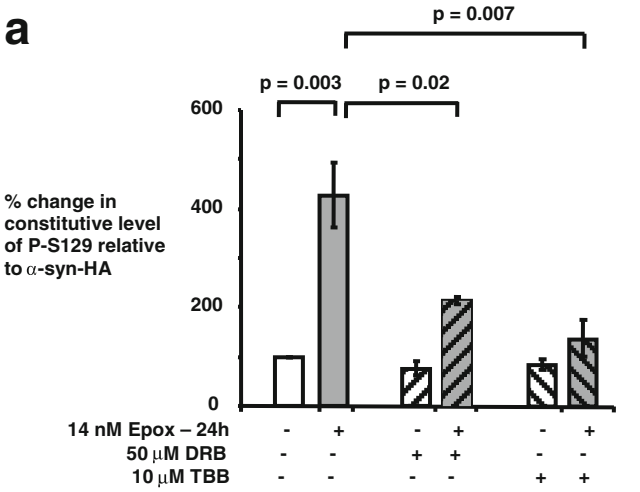


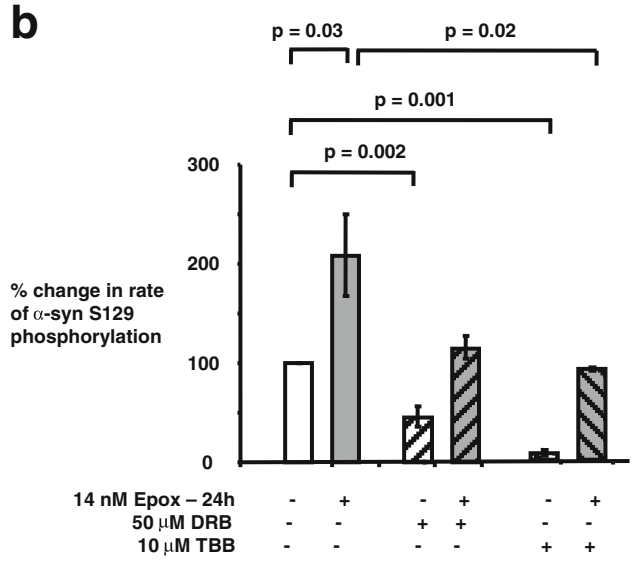
Fig. 1 PPX, in a D2/D3-independent manner, decreased the constitutive level and rate of α-synuclein phosphorylation at serine-129 (P-S129) in SH-SY5Y cells. PPX, and its enantiomer SND, decreased the constitutive level of P-S129 (against the total level of α-synuclein–HA), in a time-dependent and dose-dependent manner (**a**). Such decrease became significant (*p<0.05, **p<0.01; by ANOVA followed by Dunnett) upon 100 μM PPX/SND incubation for 48 h or longer. Shown are the mean±SEM signals of P-S129 corrected by HA of seven experiments. Typical Western blot results are shown in **b**. PPX at 100 μM (**c**) decreased the rate of P-S129, in a time-dependent manner, which reached significance (by ANOVA) by 72 h incubation. Enantiomer SND at 100 μM caused a statistically significant reduction after evaluation by *t* test. Shown are the mean±SEM signals of P-S129 corrected by HA over a period of 30 min in the presence of 1 μM OKA of 11 experiments. **d** A typical Western blot image

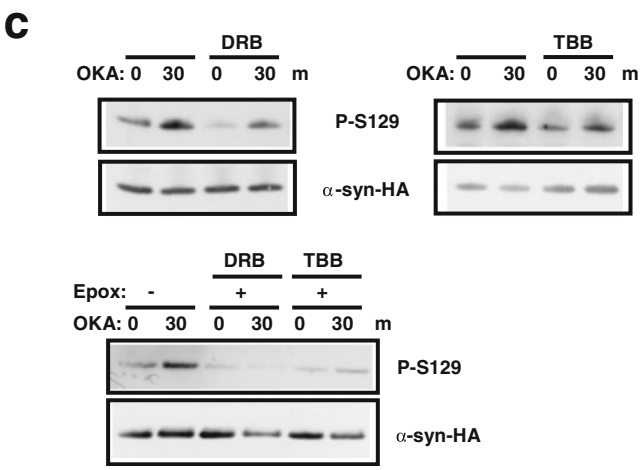












kinases examined, from the standard SelectScreen biochemical kinase profiling service (Life Technologies, Paisley, UK), inhibition was ≤10 %, including CK2, glycogen synthase kinase 3β, Akt that is downstream of phosphatidylinositol 3′-kinase and leucine-rich repeat kinase 2 (Suppl. Table). These data suggest that PPX does not inhibit the activity of

Fig. 3 Inhibition of CK2 decreased the level and rate of α-synuclein phosphorylation at serine-129 (P-S129) in SH-SY5Y cells. Specific CK2 inhibitors DRB and TBB significantly decreased the constitutive level (**a**) of P-S129 (against the total level of α-synuclein-HA), as well as the rate (**b**) (by *t* test). Shown are the mean±SEM signals of P-S129 corrected by HA of three experiments. **c** Typical Western blot images

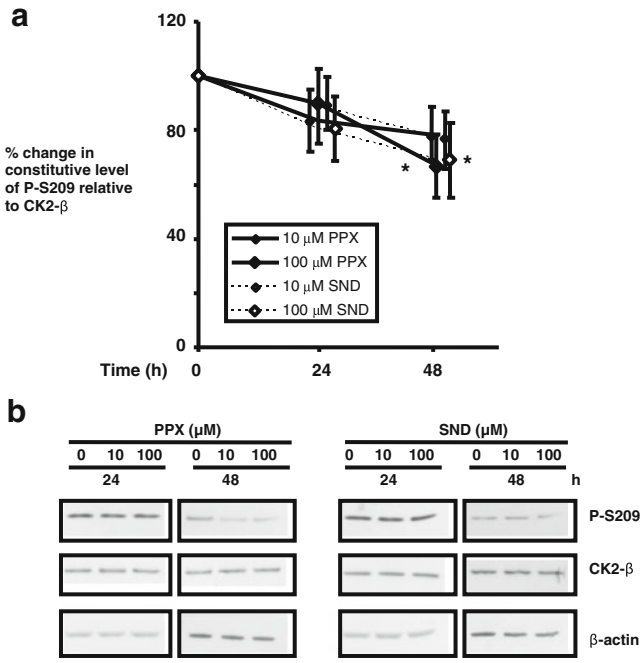


Fig. 4 D2/D3-independent decrease in the level of P-S209 CK2-β by PPX in SH-SY5Y cells. PPX, and its enantiomer SND, decreased the level of P-S209 (against the total level of CK2-β), in a time-dependent and dose-dependent (**a**) manner, significant (*$p<0.05$; by ANOVA followed by Dunnett) upon 100 μM PPX/SND incubation for 48 h. Shown are the mean±SEM signals of P-S209 corrected by the total level of CK2-β of four experiments. Typical Western blot results are shown in **b**

CK2 and/or the kinases responsible for P-S129 α-synuclein by direct interaction.

Proteasomal Activities Stimulated by Pramipexole

There are some lines of evidence that PPX affects proteasomal activities (Imamura et al. 2008; Li et al. 2010). Measurements using fluorogenic substrates on SH-SY5Y lysates enabled us to estimate proteasomal activities. Although measurements from three different substrates all produced significant reduction in activities upon epoxomicin treatment, the ability to restore the loss of proteasomal activities and to stimulate proteasomal activities by PPX or SND alone are moderate (Fig. S5A–C). We also measured the steady-state level of ubiquitination in our model, which showed a significant increase in the level of ubiquitinated protein upon epoxomicin treatment. Although not significant, PPX treatment reduced the constitutive and epoxomicin-induced level of ubiquitinated protein (Fig. S5D).

Discussion

In a human dopaminergic cell-based system with stable expression of α-synuclein, we have demonstrated that the dopamine agonist PPX can reduce both the level and rate of phosphorylation of this protein at serine-129. α-Synuclein P-S129 is the predominant form of this protein in Lewy bodies, and this post-translational modification may be important in mediating toxicity (Chau et al. 2009; Kragh et al. 2009). Both proteasomal inhibition and increased free radical generation are considered important in PD pathogenesis (Schapira 2006) and both result in an increase in P-S129 α-synuclein (Chau et al. 2009), although the underlying mechanism is not known. The dopamine receptor inactive PPX enantiomer SND demonstrated similar properties, implying that the effects on α-synuclein phosphorylation are not mediated via dopamine receptor agonist activity. This reduction in α-synuclein phosphorylation was produced through a reduction in the rate of formation of P-S129 modification, suggesting that these compounds could modulate the kinase activity involved. PPX was also effective in decreasing P-S129 formation induced by proteasomal inhibition. As illustrated in Fig. 5, we propose that PPX (in a dopamine receptor-independent fashion) stimulates proteasomal activities preventing kinase-mediated P-S129 formation and promoting turnover of P-S129 (which was shown processed by proteasome; Chau et al. 2009; Machiya et al. 2010).

In vitro experimentation has suggested that CK1 and CK2, G protein-coupled receptor kinase 2 and 5 and polo-like kinase 2 are all capable of phosphorylating α-synuclein at serine-129 (reviewed by Waxman and Giasson 2008; Mbefo et al. 2010; Braithwaite et al. 2012). We confirmed that CK2 inhibitors substantially reduce the phosphorylation of α-synuclein at serine-129, which supports that CK2 contributes, to a certain extent, to this process in our system. Our findings that PPX and its enantiomer in turn influenced

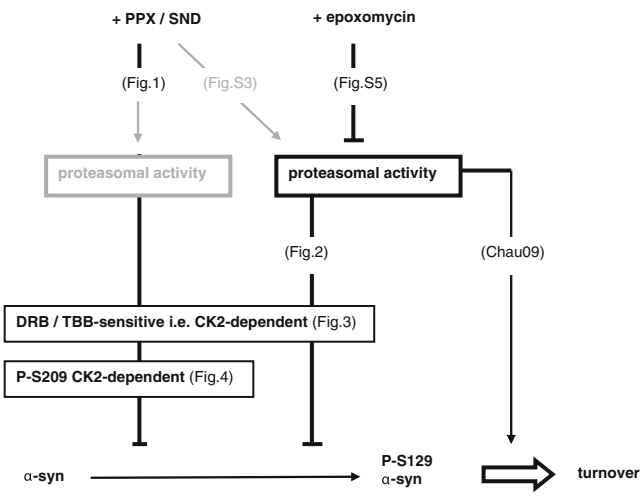


Fig. 5 Schematic diagram outlining the potential influence of PPX for P-S129 α-synuclein in this study. Items in *black* are derived from definite experimental evidence, whereas those in *grey* are descriptive

the phosphorylation of the β-subunits of CK2 at serine-209 (Bodenbach et al.1994; Litchfield 2003) provide a possible mechanism for how these compounds reduce α-synuclein phosphorylation by modulating the CK2 signalling pathway and the potential protective effect of PPX. However, our limited in vitro kinase profiling indicates that PPX did not inhibit many candidate kinases at 10 μM level.

In the same cell line used in these experiments and at similar concentrations used here, both PPX and SND have demonstrated protection against the toxins 1-methyl-4-phenylpyridinium, rotenone, paraquat, hydrogen peroxide and lactacystin (Gu et al. 2004; Iravani 2008; Ferrari-Toninelli et al. 2010). It has been proposed that PPX may exert its protective effects through several pathways, including mitochondrial protection, anti-apoptotic, anti-inflammatory and growth factor-like effects (Gu et al. 2004; Presgraves et al. 2004; Du et al. 2005; Izumi et al. 2007; Iravani et al. 2008). A protective effect has also been seen in primates in MPTP toxicity in vivo (Iravani et al. 2006) and in rodents against lipopolysaccharide-induced inflammation (Ferrari-Toninelli et al. 2010) and proteasomal inhibition (Imamura et al. 2008; Li et al. 2010). Our current finding that PPX reduced α-synuclein phosphorylation may provide a mechanistic explanation to the proteasome impairment model. It is worth investigating the neuroprotective effect of PPX in this model. The decrease in P-S129 induced by PPX adds an additional candidate pathway for the protective effects seen.

These results have several interesting implications. A double-blind randomised delayed start study of PPX in early PD has just been completed (Schapira et al. 2010). If the reduction in formation of P-S129 is a significant pathway contributing to protection, it might be expected to have a slow cumulative effect, reflecting the gradual but progressive evolution of the disease (Braak et al. 2003). The action on P-S129 does not seem to be dependent upon the activation of the dopamine receptor and so it is possible that any protection might be seen in dopaminergic and non-dopaminergic pathways, i.e. any neuron in which Lewy bodies form. This is important, as a significant component of PD morbidity is determined by non-dopaminergic degeneration. Finally, the phosphorylation pathway of α-synuclein may represent a valid target for drug discovery in PD in terms of developing additional compounds for investigation as protective agents.

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