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Alpha-synuclein aggregation and cell death triggered by energy deprivation and dopamine overload are counteracted by D_2/D_3 receptor activation

Arianna Bellucci,* Ginetta Collo,* Ilenia Sarnico,* Leontino Battistin,† Cristina Missale* and PierFranco Spano*

*Division of Pharmacology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy
†IRCCS S. Camillo Hospital, Venice, Italy

Abstract

Progressive degeneration and intraneuronal Lewy bodies made of filamentous α-synuclein (α-syn) in dopaminergic cells of the nigrostriatal system are characteristics of Parkinson's disease (PD). Glucose uptake is reduced in some of the brain regions affected by PD neurodegenerative changes. Defects in mitochondrial activity in the substantia nigra have been observed in the brain of patients affected by PD and substantia nigra lesions can induce the onset of a secondary parkinsonism. Thus, energy starvation and consequently metabolic impairment to dopaminergic neurons may be related to the onset of PD. On this line, we evaluated the effect of nutrient starvation, reproduced '*in vitro*' by glucose deprivation (GD), in primary mesecephalic neuronal cultures and dopaminergic-differentiated SH-SY5Y cells, to evaluate if decreased glucose support to dopaminergic cells can lead to mitochondrial damage, neurodegeneration and α-syn misfolding. Furthermore, we investigated the effect of dopamine (DA) treatment in the presence of a DA-uptake inhibitor or of the D_2/D_3 receptor (D_2R/D_3R) agonist quinpirole on GD-treated cells, to evaluate the efficacy of these therapeutic compounds. We found that GD induced the formation of fibrillary aggregated α-syn inclusions containing the DA transporter in dopaminergic cells. These alterations were accompanied by dopaminergic cell death and were exacerbated by DA overload. Conversely, the block of DA uptake and D_2R/D_3R agonist treatment exerted neuroprotective effects. These data indicate that glucose starvation is likely involved in the induction of PD-related pathological changes in dopaminergic neurons. These changes may be counteracted by the block of DA uptake and by dopaminergic agonist treatment.

Keywords: α-synuclein, D_2/D_3 receptor agonists, dopamine overload, dopamine transporter, glucose deprivation, Parkinson's disease.

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Loss of dopamine (DA) neurons in the nigrostriatal system (Hornykiewicz 1998) and intraneuronal proteinaceous inclusions named Lewy bodies (LB) containing aggregated α-synuclein (α-syn) (Tofaris and Spillantini 2007) are the defining histopathological features of Parkinson's disease (PD). Although a correlation exists between α-syn aggregation and degeneration of dopaminergic neurons (Tofaris and Spillantini 2007), the biochemical mechanisms regulating the dynamic and the relationship of these processes have not been clarified.

Aging is the major risk factor for the onset of idiopatic PD. Impaired energy metabolism, mitochondrial dysfunctions and increased oxidative damage have been related to the aging process in the brain (Bowling and Beal 1995). Recent findings indicate that hypoglycaemia induces exhaustion of intracellular glucose, the main source of energy for neuronal cells, leading to mitochondrial damage and to the induction of oxidative stress (Isaev *et al.* 2007). Using several different

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Address correspondence and reprint requests to Dr Arianna Bellucci, PhD, Division of Pharmacology, Department of Biomedical Sciences and Biotechnologies, University of Brescia, Viale Europa no. 11 25123, Brescia, Italy. E-mail: bellucci@med.unibs.it

Abbreviations used: α-syn, α-synuclein; D_2R/D_3R, D_2/D_3 receptor; DA, dopamine; DAT, dopamine transporter; GD, glucose deprivation; ICC, immunocytochemistry; IP, immunoprecipitation; LB, Lewy bodies; MTT, 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; PD, Parkinson's disease; PET, positron emission tomography; RT, room temperature; SDS, sodium dodecyl sulphate; SN, substantia nigra; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

methods, including positron emission tomography (PET), it has been shown that glucose uptake is impaired in several of the brain regions that are affected in the brain of PD patients (Ma and Eidelberg 2007), including the striatum. Impaired mitochondrial activity has been observed in the substantia nigra (SN) of patients affected by PD (Ambani *et al.* 1975; Kish *et al.* 1985; Marttila *et al.* 1988; Saggu *et al.* 1989; Sian *et al.* 1994; Fukae *et al.* 2007) and mitochondrial dysfunctions are known to be involved in PD pathogenesis (Schapira 2007). Moreover, focal lesions of the SN associated with metabolic impairment have been related to the onset of a form of parkinsonism whose characteristics were very similar to those of idiopatic PD (Boecker *et al.* 1996). On this line, clinical studies suggest that parkinsonian-like neurodegenerative lesions may occur in patients after strategic infarct of the basal ganglia (Reider-Groswasser *et al.* 1995). Striatal ischemic lesions can induce the down-regulation of tyrosine hydroxylase (TH) synthesis in the pars compacta neurons in an '*in vivo*' model of transient ischemia, indicating a selective sensitivity of nigral dopaminergic neurons to low brain perfusion, and consequently to energy deprivation. Furthermore, electrophysiological studies indicate that DA-containing neurons are silenced by hypoglycaemia (Guatteo *et al.* 2005) suggesting that their function may be decreased by energy deprivation. Other '*in vivo*' studies (Kitamura *et al.* 2001) demonstrated that α-syn is not scavenged in rat subjected to middle cerebral artery occlusion, indicating that the accumulation of this protein can also be induced by energy deprivation.

Taken together, these observations suggest that cell metabolism impairment may be related to the induction of α-syn misfolding and of dopaminergic neuron degeneration.

In PD, abnormal accumulation of cytoplasmic DA could also contribute to dopaminergic pathological changes (Lotharius and Brundin 2002). DA-reactive metabolites are neurotoxic (Emdadul *et al.* 2003), and DA-related oxidative stress may participate at the induction of pathological modifications of α-syn that are thought to be involved in protein misfolding (Uversky 2007). Furthermore, L-DOPA (Fahn *et al.* 2004) causes a more rapid decline in the integrity of the dopamine transporter (DAT) located in the nigrostriatal nerve terminals in the striatum, suggesting that this drug could foster the dysfunction of dopaminergic neurons. Conversely, D_2/D_3 receptor (D_2R/D_3R) agonists, showed neuroprotective affects in '*in vitro*' (Radad *et al.* 2005) and '*in vivo*' (Armentero *et al.* 2002) studies and results from PET investigations suggest that these drugs may slow the progression of the disease counteracting neuronal degeneration (Szczudlik and Rudzinska 2007).

On the basis of these observations, we aimed at studying the effect of nutrient starvation, induced '*in vitro*' by glucose deprivation (GD) on α-syn processing and folding in DA-containing neurons to evaluate whether decreased glucose support is able to induce selective deposition of α-syn and PD-like neurodegenerative changes in dopaminergic neurons. Moreover, we evaluated the effects of DA overload and D_2R/D_3R agonist treatment on these phenomena to evaluate the effects of these drugs.

To test these hypothesis we used two different '*in vitro*' models of DA neurons: primary mesencephalic rodent neuronal cell cultures and the human SH-SY5Y neuroblastoma cell line differentiated into a dopaminergic-like phenotype (Presgraves *et al.* 2004).

Our data indicated that glucose starvation is able to induce PD-like pathological changes in dopaminergic cells. Indeed, we found that GD reduced cell viability and induced selective α-syn increase and aggregation into intracytoplasmic inclusions also containing the DAT in dopaminergic cells. These effects were exacerbated by increased intracellular DA overload. The D_2R/D_3R agonist quinpirole and the DA-uptake blocker cocaine counteracted α-syn aggregation and exerted neuroprotective effects.

These results are relevant for the understanding of the molecular mechanisms underlying PD-related neurodegeneration and for the development of novel therapeutic strategies for this and other LB-related disorders.

Methods

Cell cultures

Primary embryonic mouse ventral mesencephalic cells were isolated and cultured according to the method described by Studer (1997) with minor modifications. Briefly, ventral mesencephalic tissues were dissected from embryonic day 12.5 mice and were incubated in Dulbecco's phosphate-buffered saline (PBS) containing Accumax (Innovative Cell Technologies Inc., San Diego, CA, USA) and 50 μg/mL deoxiribonuclease (Sigma-Aldrich, Milan, Italy) for 10 min at 25°C. After mechanical dissociation using sterile micropipette tips, the single cell suspension was resuspended in Neurobasal medium (Gibco, Milan, Italy) and centrifugated. Cell count and viability assay was performed using the trypan blue exclusion test at 1000 *g*. Cells were seeded on poly-D-lysine and laminin-coated coveslides (4×10^4 cells per coverslide) or on poly-D-lysine and laminin-coated Petri dishes (5×10^5 cells per well). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2 in Neurobasal medium containing 2 mM glutamine (EuroClone, Milan, Italy) and 1% B27 supplement (Gibco). Fifty percentage of the medium was changed every second day until treatment. Five-day-old cultures were used for treatment. All experiments were carried out according to the ECC guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). All efforts were made to minimize animal suffering.

SH-SY5Y cells (ATCC, Manassas, VA, USA) were terminally differentiated toward a dopaminergic phenotype as previously described (Pennypacker *et al.* 1989; Presgraves *et al.* 2004). Differentiated cells were conventionally named SH-SY5Y$^+$ cells. Undifferentiated cells were used as controls. Briefly, cells were grown to confluence in complete medium made up by Dulbecco's modified

Eagle's medium supplemented with 10% of heat-inactivated new born calf serum, 100 μg/mL penicillin, 100 μg/mL streptomycin and 0.01 μM non-essential amino acids (Gibco). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. For immunocytochemistry (ICC), 3-[4,5-dimethylthiazol]-2,5-diphenyl-tetrazolium (MTT) assay and western blotting (WB) analysis, cells were seeded (2×10^4 cells/cm^2) either on collagen-coated slides or 24 well plates or in Petri dish respectively. Differentiation was performed incubating the cells for 3 days in complete medium supplemented with 10 μM of *all-trans*-retinoic acid (Sigma-Aldrich) and for the following 3 days in complete medium containing 80 nM of 12-*O*-tetradecanoyl-phorbol-13-acetate.

Glucose deprivation and dopamine treatment

Mouse ventral mesencephalic cells were treated after 5 days *in vitro*. At the time of treatment, ICC analysis indicated that the mouse ventral mesencephalic cells were composed of 99.1% neurons, 5% of which were TH- and DAT-immunoreactive dopaminergic neurons and 0.9% astrocytes. GD was performed through an incubation of the cells with Hank's balanced salt solution (Sigma-Aldrich) supplemented with 2 mM glutamine and 1% of B27 was performed for 1 h at 37°C. The cultures were incubated with DA (Sigma-Aldrich) for 4 h at 37°C, then with the usual medium for 24 h and fixed.

For neuroblastoma cell line SH-SY5Y, GD was performed following the same protocol as referred for primary mesencephalic cultures with minor modifications regarding a decrease in the time of GD lesion because of the higher metabolism rate of this cell line with respect of primary neurons. Briefly, SH-SY5Y$^+$ and SH-SY5Y cells were incubated for 15 min at 37°C in medium without glucose made by Hank's balanced salt solution (Sigma-Aldrich) supplemented with 10% of new born calf serum and 0.01 μM non-essential amino acids.

Then this medium was removed and replaced either with complete medium for 24 h or with complete medium plus DA (Sigma-Aldrich) (containing 5×10^{-5} M DA) for the following 4 h and then with complete medium without DA for 20 h. For ICC and WB studies, cells were fixed or scraped at 24 h from GD respectively. For thioflavin-s and α-syn double staining cells were fixed right after ($t = 0$) or 5 ($t = 5$), 15 ($t = 15$), 24 ($t = 24$) or 48 ($t = 48$) h after GD.

To exclude the effects of media substitutions control cells were subjected to media changes at the beginning and at the end of the GD insult of treated cells, as well as at 4 h from GD in the case of the controls of GD + DA-treated cells.

Drug treatments

Cocaine 5×10^{-5} M (Sigma-Aldrich), quinpirole 5×10^{-5} M (Sigma-Aldrich), L-sulpiride 5×10^{-5} M (Tocris biosciences, Bristol, UK) and spiperone 5×10^{-5} M (Tocris Bioscience) were administered to the cells directly adding them to complete medium 15 min before the addiction of DA to achieve a good blockade of the DAT or to pre-stimulate or pre-inhibit D_2R/D_3R. Drugs were maintained in the medium for 4 h during DA incubation.

3-[4,5-Dimethylthiazol]-2,5-diphenyltetrazolium assay

The cytotoxic effect of GD and DA incubation was measured by evaluating mitochondrial dehydrogenase activity using the MTT salt assay as described by the manufacturer's instructions (Sigma-Aldrich). Briefly, the MTT stock solution (5 mg/mL) was added to each well at one-tenth the total media volume and left to incubate at 37°C for 4 h. After this incubation, the medium was removed and the converted dye was solubilized with dimethylsulphoxide for 2 h. Absorbance of the dye was measured at 570 nm with a background subtraction at 670 nm. Each experiment was performed in triplicate and repeated at least twice. The data were totalled and averaged for each of three replications. Data were expressed as percentage cell viability with respect to the control untreated cells, plotted on graph and analysed.

Immunocytochemistry analysis

At the end of the experiments, cells were fixed by incubation for 15 min in 3% *p*-formaldehyde/3% sucrose made up in PBS 1 M, pH 7.4, and then stored in PBS containing 0.05% sodium azide. Slides were incubated for 30 min at 25°C in 5% (v/v) normal goat serum plus 2% (w/v) bovine serum albumin in PBS containing 0.3% Triton X-100, then overnight at 4°C with primary antibody at the optimal working dilution made up in PBS containing 1% normal goat serum. On the following day, cells were then incubated for 2 h at 25°C with the fluorescent secondary antibody diluted in PBS containing 0.3% Triton X-100. For double labelling, at the end of this incubation, cells underwent another cycle of staining. Cell nuclei were counterstained with Hoechst (Sigma-Aldrich). Coverslips were then mounted on glass slides with mowiol-dabco (Sigma-Aldrich) and observed by means of an epifluorescence microscope (Olympus IX50; Olympus, Milan, Italy). To confirm the colocalization of DAT and α-syn within dopaminergic cells the sections were observed by means of a Zeiss confocal laser microscope (Carl Zeiss S.p.A., Milan, Italy), with the laser set on λ = 488–543 nm and the height of the sections scanning = 1 μm. Images (512 × 512 pixels) were then reconstructed using LSM Image Examiner (Carl Zeiss S.p.A) and Adobe Photoshop 7.0 (Adobe system, Mountain View, CA, USA) software.

Antibodies

Alpha-synuclein was visualized by using the following monoclonal antibodies: α-syn 211 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and syn-1 (BD-Bioscience, Milano, Italy) recognizing residues 121–125 of the human form and residues 91–99 of the human and rat form (Perrin *et al.* 2003) of α-syn respectively. Anti-DAT (Santa Cruz Biotechnology) and anti-TH (Chemicon, Temecula, CA, USA) polyclonal antibodies were used to visualize the respective substrates.

Thioflavin-S/α-synuclein double staining

For thioflavin-S staining, cells were incubated with 0.05% of thioflavin S (Sigma-Aldrich) in PBS, washed three times for 10 min in 80% EtOH and then blocked for subsequent immunostaining with the α-syn 211 antibody as previously described. Cell Nuclei were counterstained with Hoechst (Sigma-Aldrich).

For counting thioflavin-S-/α-syn-positive inclusions at different time points after GD ($t = 0$, $t = 5$, $t = 15$, $t = 24$, $t = 48$ h after GD), stained coverslips were observed by a rater blinded to experimental conditions using standard epifluorescence (100× magnification). Four coveslips (20 fields each) per type were observed and assessed for the number of thioflavin-S-positive α-syn inclusions per cell.

Western blotting and immunoprecipitation studies

For DAT, TH and total α-syn extraction cells were lysed in Tris-buffered saline $(TBS)^+$ (50 mM Tris–HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM *N*-ethylmaleimide, plus complete proteasome inhibitor mixture; Roche Diagnostics, Mannheim, Germany). Protein concentration in the samples were measured by using the Bradford assay (Pierce, Rockford, IL, USA). Equal amounts of proteins (20–25 μg) were run on 4–12% NuPAGE Novex Bis–Tris gels (Invitrogen, Milan, Italy). Densitometric analysis of bands was performed by means of Gel Pro Analyzer version 6.0 (MediaCybernetics, Bethesda, MD, USA). All bands were normalized to α-tubulin levels as a control of equal loading of samples.

For soluble or detergent-insoluble α-syn collection sequential extraction in TBS^+, Triton X-100, radioimmunoprecipitation (RIPA) buffer and 8 M urea/5% sodium dodecyl sulphate (SDS) was performed with a protocol adapted from the one described by Tofaris *et al.* 2006 (Tofaris *et al.* 2006). Briefly cells were lysed in TBS^+ and spun for 30 min at 120 000 *g* at 4°C, with the resulting supernatants corresponding to TBS^+ soluble fraction. Pellets were then homogenised in TBS^+ containing 1% of Triton X-100 followed by centrifugation at 1000 *g*. Supernatants were discarded and the resulting pellets were homogenised in RIPA buffer (50 mM Tris–HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 1% NP-40 (Sigma-Aldrich) and 0.5% sodium deoxycholate) and 0.1% SDS followed by centrifugation at 1000 *g*. The detergent-insoluble pellets were then reconstituted in 8 M urea/5% SDS. For gel analysis samples were run on 4–12% NuPAGE Bis–Tris gels (Invitrogen), blotted on nitrocellulose membranes and probed with the opportune antibody.

For immunoprecipitation (IP) studies equal amounts of total protein extracts (80 μg) were incubated overnight, under agitation at 4°C, in ice-cold radioimmunoassay buffer (400 mM NaCl, 20 mM EDTA, 20 mM Na_2HPO_4 and 1% NP-40), 0.1% SDS and synuclein 211 (Santa Cruz Biotechnology) or syn-1 (BD-Bioscience) diluted 1 : 50. The following day, after adding Protein A/G Plus Agarose beads (Santa Cruz Biotechnology), the samples were incubated under agitation for 3 h at 25°C and then centrifuged at 1000 *g* for 1 min at 4°C. Pellets were washed three times with RIPA buffer and then homogenized in 50 μL of sample buffer. For gel analysis, samples were boiled for 5 min, run on a 10% SDS–polyacrylamide gel electrophoresis gel, blotted on a nitrocellulose membrane and probed with DAT antibody (Santa Cruz Biotechnology).

Statistical analysis

Differences in DAT and α-syn total levels and cell viability between control SH-SY5Y and $SH\text{-}SY5Y^+$ cells after DA, GD and GD + DA treatments as well as statistical analysis of thioflavin-S/α-syn-positive inclusions in differentiated cells after GD and GD + DA treatment were assessed by two-way ANOVA followed by Bonferroni's multiple comparison tests for pair-wise comparisons. One-way ANOVA followed by Bonferroni's post-comparison test was used to evaluate the differences between total α-syn and DAT levels of primary mesencephalic neurons after GD and GD + DA treatments and to evaluate the effects of cocaine, quinpirole, spiperone and L-sulpiride treatments on GD + DA-treated differentiated cells. All calculations were performed using GraphPad Prism version 4.00 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA). Statistical significance was established at $p < 0.05$.

Results

GD induces the formation of α-syn inclusions containing the DAT and affects α-syn and DAT levels in primary mesencephalic neurons

The effect of GD on α-syn folding was investigated in mouse primary mesencephalic neuron cultures.

Double labelling ICC showed that DAT-positive primary mesencephalic neurons (Fig. 1b) were also α-syn-positive (Fig. 1a); α-syn staining was distributed in the cell bodies and throughout the processes (Fig. 1a) of DAT-positive neurons as confirmed by the merge of the two immunostainings (Fig. 1c).

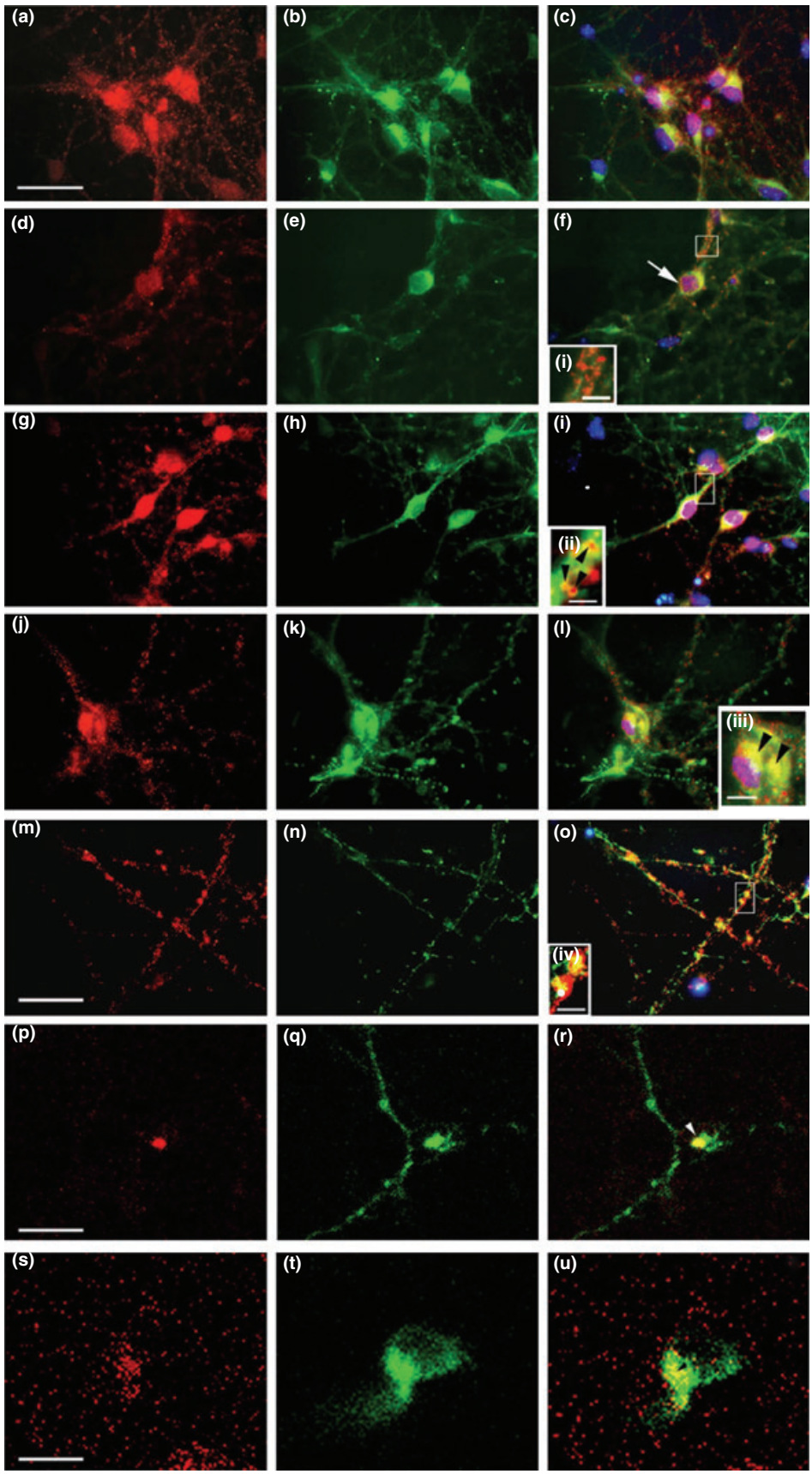
As reported in Fig. 1(d–f), DA treatment led to an increase of α-syn and DAT membrane staining in the cell body (arrow) and processes [Fig. 1f(i)]. These data indicate that DA stimulates both DAT and α-syn translocation to the plasma membrane, suggesting that the two protein may share common trafficking mechanisms that are regulated by DA.

After GD (Fig. 1g–i), α-syn and DAT stainings were condensed in dot-like inclusions in the cell bodies (Fig. 1i) of primary dopaminergic neurons, suggesting the occurrence of protein aggregation.

Recent studies indicate that DA- and thus L-DOPA-derived toxic metabolites, produced in oxidative stress conditions, are able to interact with α-syn protofibrils and to induce filamentous α-syn accumulation '*in vitro*' (Uversky 2007). We thus evaluated the effect of DA overload on α-syn accumulation in cultured DAT-positive mesencephalic neurons after GD. The effect of DA overload combined with GD on α-syn- and DAT-positive inclusion formation is reported in Fig. 1(j–r). DA treatment increased the formation of α-syn aggregates induced by GD in the cell bodies (Figure 1j–l(iii)). Moreover, we observed that in the GD + DA-treated cells, α-syn (Fig. 1m) and DAT (Fig. 1n) stainings were colocalized (Fig. 1o(iv)) in round-shaped inclusions also within neuronal processes.

The formation of α-syn-DAT protein inclusions was also confirmed by confocal analysis of GD + DA-treated primary dopaminergic neurons (Fig 1m–r). Indeed, Fig 1 shows the presence of α-syn-immunopositive inclusions containing the DAT in a process (Fig. 1p–r) and in the cell body (Fig. 1s–u) of dopaminergic neurons subjected to GD + DA treatment.

The western blot analysis reported in Fig. 2 shows that exposure of mesencephalic neurons to both GD and GD combined with DA overload not only induced α-syn aggregation, but also resulted in increased α-syn (marked by the SYN-1 antibody) (Fig. 2a and c) and decreased DAT (Fig. 2b and d) expression. Conversely, DA treatment by itself did not influence α-syn and DAT levels. These observations suggest that GD may modulate the function of dopaminergic cells also by regulating α-syn and DAT expression.



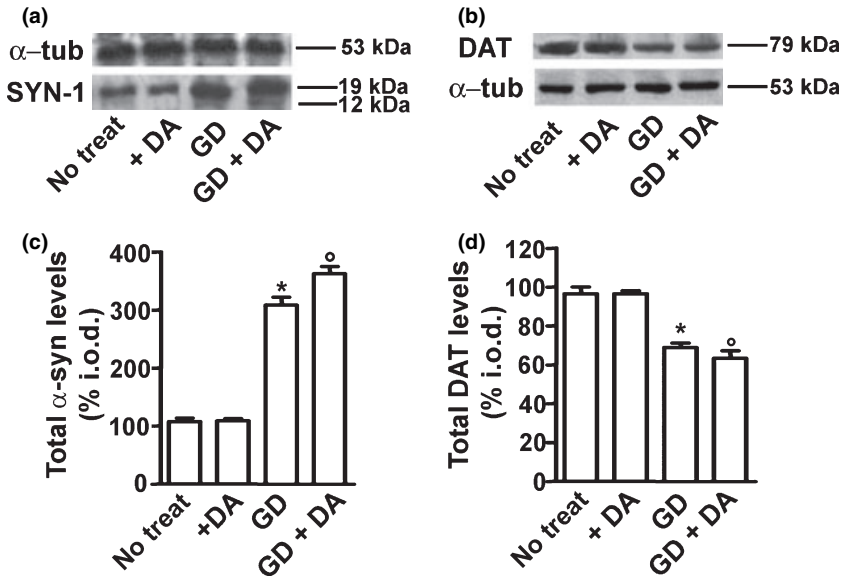


Fig. 2 (a and b) Images of SYN-1 (p) and DAT (q) WB of untreated, GD- and GD + DA-treated cells, showing an increase in α-synuclein levels and a decrease in DAT levels in GD- and GD + DA-treated primary mesencephalic neurons. α-Tubulin WB is reported as a control for equal loading of the different protein samples. (c) Quantitative analysis of total α-synuclein levels (SYN-1 antibody) in untreated and GD- and GD + DA-treated mesencephalic neurons. *+200.5% ($p < 0.01$) and °+254.5% ($p < 0.01$) versus untreated cells; $n = 5$ for each group. (d) Quantitative analysis of total DAT levels in untreated and GD- and GD + DA-treated mesencephalic neurons. *−27.55% ($p < 0.05$) and °−33.15% ($p < 0.05$) versus untreated cells. $n = 5$ for each group.

GD and DA treatment reduced cell viability of differentiated SH-SY5Y cells

Because of the low density of dopaminergic cells in the primary mesencephalic cultures and the heterogeneity of this preparation, we developed a homogeneous dopaminergic neuronal model. In particular, the human SH-SY5Y neuroblastoma cell line differentiated into a dopaminergic phenotype by *all-trans*-retinoic acid/12-*O*-tetradecanoyl-phorbol-13-acetate treatment (SH-SY5Y$^+$) was used to characterize the molecular mechanisms involved in the formation of α-syn/DAT-positive intracellular aggregates. Indeed, SH-SY5Y$^+$ cells represent a good and reproducible '*in vitro*' model of DA-like neurons as they express high levels of dopaminergic neuronal markers, such as DAT and TH and high α-syn levels. DAT-dependent DA-uptake is also increased in SH-SY5Y$^+$ cells with respect of control undifferentiated cells (please see supplemental data for more information). Moreover, previous findings demonstrated that differentiated SH-SY5Y cells also express high levels of D_2R and D_3R and respond to dopaminergic agonist treatment (Presgraves *et al.* 2004).

The effect of GD, GD + DA and DA treatments on SH-SY5Y$^+$ cell viability was evaluated by means of the MTT assay (Fig. 3) which is also indicative of mitochondrial

Fig. 1 Double immunofluorescence labelling for α-synuclein (α-syn; red staining) and DAT (green staining) on mouse primary mesencephalic neurons. (a–c) Representative photomicrographs showing α-syn (a) and DAT (b) double staining in primary dopaminergic mesencephalic neurons. Note the partial colocalization of the two immunoreactivities within cell bodies and processes of neurons indicated by the yellow colour in the merge (c) of the two images. (d–f) DA treatment increased both α-syn (d) and DAT (e) immunoreactivities at the plasma membrane. This effect was evident in the cell body (arrow) and in the processes, where the two staining appeared to colocalize as indicated by the yellow-orange colour in the merge (f). [f(i)] Note the presence of intense DAT and α-syn staining at the plasma membrane in the cell processes. (j–l) α-syn staining (j) and DAT (k) staining in GD + DA-treated cells. Note the colocalization of the proteins within intracytoplasmic inclusions [l, arrows in l(ii)] in a primary dopaminergic neuron. (m–o) Following GD + DA insult α-syn (m) and DAT (n) colocalized in cell processes as indicated by the yellow colour of the merge in (o). Note the presence of round-shaped DAT- and α-syn-positive inclusions within cell processes of primary neurons after GD and DA treatment in the inset in (o) showing higher magnification of the area selected by the rectangle. (p–r) Confocal images of an α-syn (p)-/DAT(q)-immunopositive inclusion in the cell process of a mesencephalic dopaminergic neuron following GD + DA treatment. Note the colocalization of the two stainings within the inclusion indicated by the arrowhead in (r). (s–u) Representative photomicrographs showing confocal α-syn (s) and DAT (q) immunostainings in the cell body of a mesencephalic dopaminergic neuron following GD + DA treatment. Note the colocalization of the two staining within an intracytoplasmic inclusion indicated by the arrowhead in (u). Scale bars: 50 μm for (a–l); 6 μm for [f(i)]; 4 μm for [i(ii) and l(iii)]; 20 μm for (m–o); 2 μm for the [o(iv)]; 10 μm for (p–r); 4 μm (s–u).

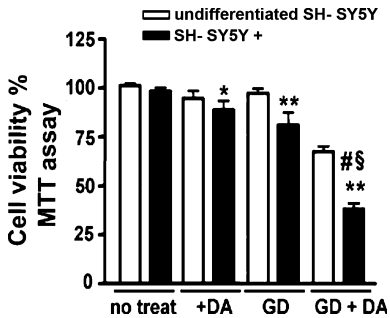


Fig. 3 Percentage changes of cell viability of undifferentiated (white bars) and (black bars) SH-SY5Y$^+$ cells in different experimental conditions assayed by the MTT method. Two-way ANOVA revealed a statistically significant effect of DA, GD and GD + DA treatments ($F_{3,40} = 29.17$, $p < 0.0001$) and cell differentiation ($F_{1,40} = 51.69$, $p < 0.0001$) on cell viability as revealed by the significant interaction between these two parameters ($F_{3,40} = 12.27$, $p < 0.0001$). Cell viability of differentiated cells were significantly decreased after DA, GD and GD + DA treatments (*−16.22%, $p < 0.01$; **−29.85%, $p < 0.001$, **−59.33%, $p < 0.001$) with respect to differentiated untreated cells. GD + DA-treated differentiated cells showed a statistically significant reduction of 49.48% ($^{\#}p < 0.001$) and 43.11% ($^{\S}p < 0.001$) in comparison with DA- and GD-treated cells. GD followed by 4 h DA incubation was able to induce a statistically significant reduction of 12.93% ($p < 0.05$) of cell viability of control cells with respect to untreated control cells; $n = 10$ for each group.

dysfunction. GD induced a significant decrease of SH-SY5Y$^+$ cell viability. When GD was followed by DA overload SH-SY5Y$^+$ cell viability was further reduced in comparison with glucose deprived-SH-SY5Y$^+$ cells suggesting that DA treatment exacerbates the mitochondrial impairment/neurodegenerative effect brought about by GD. DA treatment by itself also slightly decreased SH-SY5Y$^+$ cell viability. No changes in cell viability were observed in undifferentiated SH-SY5Y cells exposed to either DA or GD. On the other hand, GD followed by 4 h DA incubation also induced a statistically significant reduction of the cell viability of undifferentiated cells. These results suggest that GD is toxic for dopaminergic differentiated cells and that DA treatment exacerbates the neurodegenerative effects bought about by GD, indicating a toxic effect of this biogenic amine in metabolic stress conditions.

GD and DA overload induce the formation of α-syn-/DAT-positive inclusions in differentiated SH-SY5Y cells

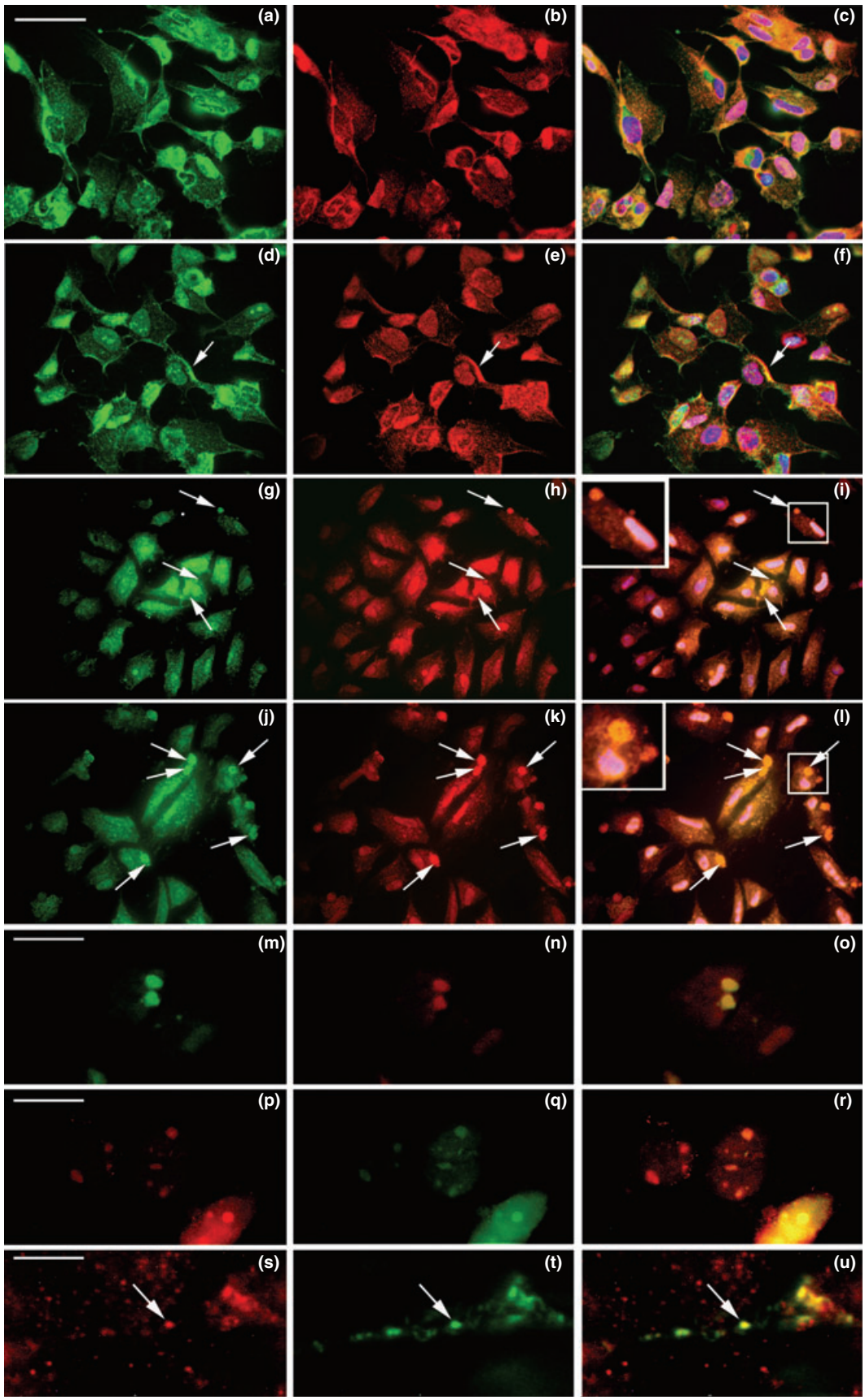
According to the results obtained in primary mesencephalic neurons, after GD we observed the formation of intracellular α-syn- and DAT-immunopositive inclusions in SH-SY5Y$^+$ cells. Indeed, immunocytochemical studies showed that in SH-SY5Y$^+$ cells DAT (Fig. 4g) and α-syn (Fig. 4h) immunoreactivities were distributed and partially colocalized (Fig. 4i) in the cell bodies and processes. In DA-treated SH-SY5Y$^+$ cells, we observed that both DAT and α-syn immunostainings were mainly localized at the plasma membrane (arrows in Fig. 4d–f) suggesting that DA promotes the membrane translocation of both proteins. These effect is in agreement with the results obtained by using mouse primary dopaminergic cells.

In SH-SY5Y$^+$ cells, GD induced the translocation of both α-syn and DAT in round-shaped aggregates at cytoplasmic level as indicated by the arrows in Fig. 4g–i and shown in the higher magnification panel in Fig. 4i. When GD was followed by a 4 h DA treatment DAT and α-syn double stained inclusions were also observed within cell cytoplasm (indicated by the arrows in Fig. 4j–l) as shown in the insert in Fig. 4l.

The presence of α-syn-/DAT-positive inclusions in glucose deprived SH-SY5Y$^+$ cells was also confirmed by confocal microscopy. Indeed, Fig. 1 shows DAT and α-syn immunopositive inclusions within the cell bodies of SH-SY5Y$^+$ cells subjected to GD (Fig. 1m–o) and GD + DA (Fig. 1p–r) treatment. Moreover, by confocal microscopy we also observed the presence of α-syn-/DAT-positive inclusions within some of the cell processes of dopaminergic differentiated SH-SY5Y cells as shown in Fig. 1(s–u).

No changes in DAT or α-syn staining and localization were observed in undifferentiated cells in basal conditions (Fig. 5a–c) and after DA (Fig. 5d–f), GD (Fig. 5g–i) and GD + DA (Fig. 5j–l) treatments.

Fig. 4 DAT (green) and α-synuclein (red) double staining in SH-SY5Y$^+$ neuroblastoma cells in basal conditions (a–c), after DA treatment (d–f), after GD (g–i) and after GD + DA treatment (j–l). (a–c) Images showing DAT (a) and α-synuclein (b) staining in SH-SY5Y$^+$ cells. Note the colocalization of the staining within the cell processes of these cells (c). (d–f) Representative photomicrographs showing that DA treatment increased both DAT (d) and α-synuclein (e) immunoreactivities at the plasma membrane. Note the colocalization of the two stainings at the plasma membrane (arrow) showed by the yellow colours in the merge of the two images (f). (g–i) GD induced the formation of intracellular inclusions that were immunopositive for DAT (g) and α-synuclein (h) within the cell cytoplasm (indicated by the arrows). Note the colocalization of DAT and α-synuclein staining within the round-shaped inclusions (inset in i). (j–l) DAT (j) and α-synuclein (k) double staining after GD + DA treatment, revealed the presence of several inclusions that were immunopositive for both proteins (indicated by the arrows) within the cell bodies. Note the round-shaped morphology of the inclusions (inset in l). (m–o) Confocal images of two α-synuclein-/DAT-immunopositive inclusions within the cell cytoplasm of a glucose-deprived SH-SY5Y$^+$ cell. (p–r) Confocal images of α-synuclein-/DAT-immunopositive inclusions within the cell cytoplasm of SH-SY5Y$^+$ cells subjected to GD + DA treatment. (s–t) Confocal images showing the presence of an α-synuclein-/DAT-positive inclusion (arrows) within a cell process of a SH-SY5Y$^+$ cell after GD + DA treatment. Scale bars: 110 μm for (a–l); 40 μm for the insets in (i and l); 40 μm for (m–o); 50 μm for (p–r); 15 μm for (s–u).



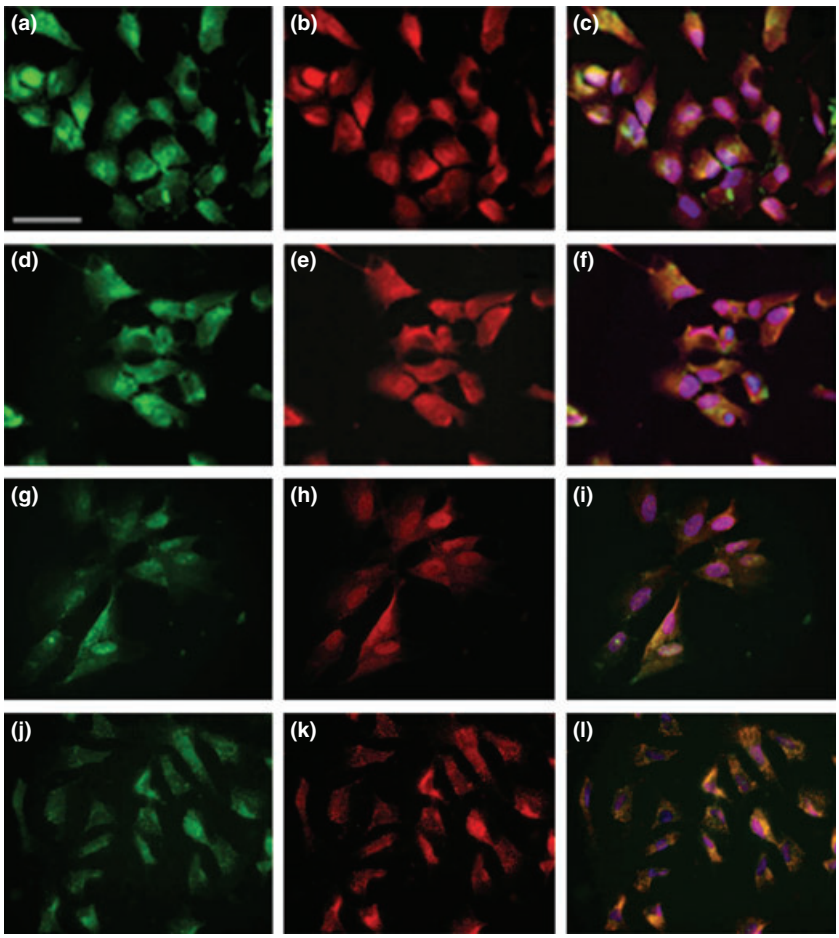


Fig. 5 DAT (green) and α-synuclein (red) double staining in undifferentiated SH-SY5Y neuroblastoma cells in basal conditions (a–c), after DA treatment (d–f), after GD (g–i) and after GD + DA treatment (j–l). (a–c) Representative photomicrographs showing DAT (a) and α-synuclein (b) double staining (c) in undifferentiated neuroblastoma cells. Both DAT and α-synuclein staining are mainly distributed within the cell cytoplasm. (d–f) DAT (g) and α-synuclein (h) staining in DA-treated undifferentiated cells did not affect the subcellular distribution of the two proteins with respect of untreated SH-SY5Y cells. DAT and α-synuclein stainings are distributed throughout the cell cytoplasm. (g–i) GD did not affect DAT (g) and α-synuclein (h) subcellular localization and did not change the localization of the two proteins (i) within the cell cytoplasm in undifferentiated neuroblastoma cells. (j–l) Images are showing DAT (j) and α-synuclein double staining (k) in undifferentiated SH-SY5Y cells subjected to GD and DA treatment. On these cells, the hypoglycaemic insult did not induced any change in the cellular distribution of the two stainings. Scale bars: 110 μm for (a–l).

These data indicate that SH-SY5Y$^+$ cells represent a reliable '*in vitro*' model to be used in parallel with mouse primary mesencephalic neurons to study the neurodegenerative changes involving DAT and α-syn induced by GD and DA overload and to characterize α-syn and DAT-positive inclusions at the molecular level.

DAT and α-syn directly interact in SH-SY5Y$^+$ cells and their levels are significantly changed by GD and DA overload

Recent studies indicate that α-syn may directly interact with DAT (Lee *et al.* 2001; Wersinger *et al.* 2003) and that the increase of α-syn levels correlates with high risk of protein aggregation (Tofaris and Spillantini 2005). As we observed that DAT and α-syn are colocalized and traffick together in dopaminergic cells, we evaluated whether GD affects DAT and α-syn expression levels and interaction and induces biochemical changes, such as carboxyl-terminal cleavage of α-syn.

Dopamine transporter and α-syn levels were measures by WB and quantified by densitometric analysis. Total (Fig. 6a) and membrane-bound (Fig. 6b) DAT levels in the immunoblottings were markedly reduced after GD treatment in SH-SY5Y$^+$ cells but not in control cells. When GD was followed by 4 h DA incubation, a further decrease of total and membrane-bound DAT levels was observed only in differentiated cells, indicating that DA exacerbates the biochemical changes brought about by GD. These data were further confirmed by densitometric analysis of total (Fig. 6g) and membrane-bound (Fig. 6h) DAT-immunopositive bands showing a significant reduction of the transporter in SH-SY5Y$^+$ cells after GD and GD + DA treatment. DA treatment by itself did not change total DAT levels in SH-SY5Y$^+$ cells, indicating that the effects may be closely dependent on the stress conditions induced by GD. Conversely, DA treatment statistically increased membrane-bound DAT levels in SH-SY5Y$^+$ cells, indicating that this amine favours the membrane translocation of the transporter.

To evaluate whether GD and DA overload may induce pathogenic α-syn cleavage we performed WB analysis of whole cell homogenates using two distinct antibodies: SYN-211 directed against the carboxy-terminal part of α-syn, and recognizing full-length α-syn, and SYN-1, directed against an intermediate portion of α-syn and recognizing both full-length and carboxy-terminal cleaved form of the protein. An increase in SYN-211 (Fig. 6c) and SYN-1 (Fig. 6d) immunoreactivities was observed in SH-SY5Y$^+$ cells after GD and

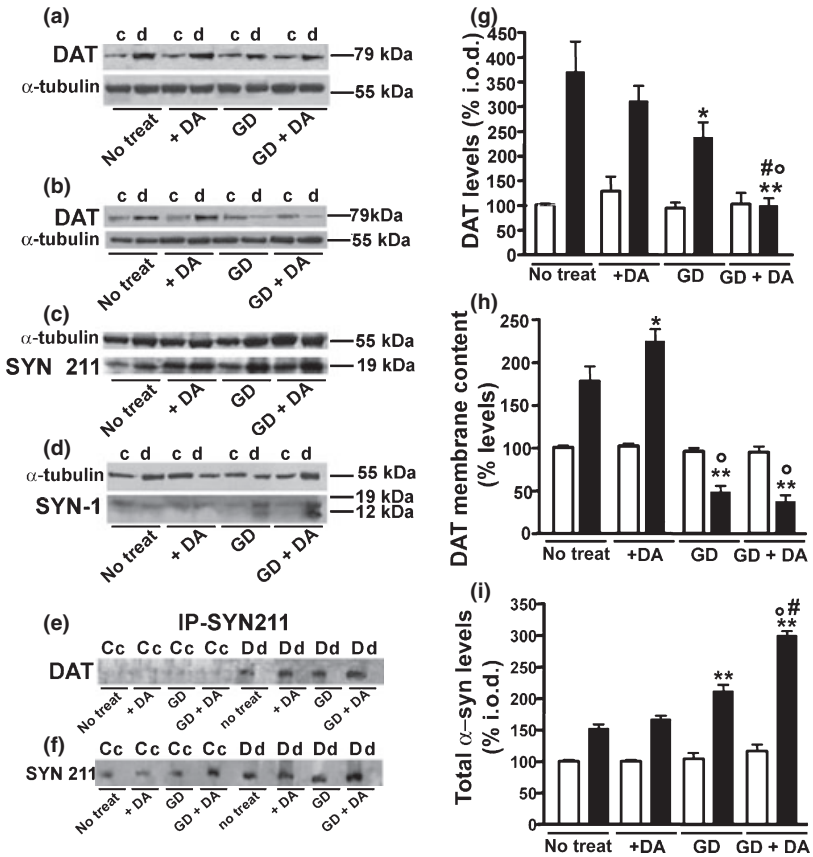


Fig. 6 Representative photomicrographs showing immunoblottings for total (panel a) and membrane-bound (panel b) DAT, SYN-211 (panel c) and SYN-1 (panel d) levels of untreated (no treat) and DA- (+DA), GD- and GD + DA-treated SH-SY5Y cells. 'c' undifferentiated cells and 'd' SH-SY5Y$^+$ cells. GD- and GD + DA-treated differentiated cells showed a decrease of total (a) and membrane-bound (b) DAT levels in parallel with an increase in total α-synuclein (c and d) levels. Note the presence of carboxy-terminal cleaved species of α-synuclein in the immunoblot for SYN-1 antibody (panel d). (e) Immunoblotting for SYN-211 of DAT immunoprecipitates of untreated (no treat) and DA- (+DA), GD- and GD + DA-treated, undifferentiated 'C' and SH-SY5Y$^+$ 'D' cells. 'c' control samples without AB and 'd' control samples without AB. (f) Immunoblotting for SYN-211 on the SYN-211 immunoprecipitates used for the DAT blotting of panel (e). 'C' undifferentiated SH-SY5Y cells, 'c' control samples without AB; 'D' SH-SY5Y$^+$ cells, 'd' control samples without AB. (g) Total DAT levels in undifferentiated (white bars) and SH-SY5Y$^+$ (black bars) cells in basal (no treat) conditions and after DA, GD and GD + DA treatments. WB analysis revealed a decrease in DAT immunoreactivity in SH-SY5Y$^+$ cells, while no changes in control cells, after GD and GD followed by DA treatment. Densitometric analysis of DAT-immunopositive bands revealed a significant interaction ($F_{3-27} = 5.565$, $p < 0.01$) between treatments and cell differentiation with a statistically significant different effect of treatments ($F_{3-27} = 5.593$, $p < 0.01$) on control and differentiated SH-SY5Y cells and a significant difference between the effect of treatments on both cellular phenotypes ($F_{1-27} = 24.31$, $p < 0.001$). *−132.9% ($p < 0.05$) and **−270% ($p < 0.001$) versus untreated SH-SY5Y$^+$ cells. DA treatment following GD insult is able to exacerbate the reduction of DAT expression levels with respect to GD- ($^{\#}$−137.8% $p < 0.05$) and to DA-treated (°−161% $p < 0.05$) differentiated cells respectively; $n > 10$ for each group. (h) DAT membrane content in undifferentiated (white bars) and SH-SY5Y$^+$ (black bars) cells in basal (no treat) conditions and after DA, GD and GD + DA treatments. Densitometric analysis of DAT-immunopositive bands revealed a significant interaction ($F_{3-15} = 38.28$, $p < 0.0001$) between treatments and cell differentiation with a statistically significant different effect of treatments ($F_{3-15} = 45.08$, $p < 0.0001$) on control and differentiated SH-SY5Y cells and a significant difference between the effect of treatments on both cellular phenotypes ($F_{1-15} = 6.74$, $p < 0.05$). **−129.3, $p < 0.001$ and −183.8, $p < 0.001$ for GD- and GD + DA-treated SH-SY5Y$^+$ cells, respectively, versus untreated SH-SY5Y$^+$ cells. °−197.5, $p < 0.001$ and °−203.8, $p < 0.001001$ for GD- and GD + DA-treated SH-SY5Y$^+$ cells, respectively, versus untreated SH-SY5Y$^+$ cells. *+30%, $p < 0.05$ for DA-treated versus untreated SH-SY5Y$^+$ cells. (i) Total α-synuclein levels in undifferentiated (white bars) and SH-SY5Y$^+$ (black bars) cells in basal (no treat) conditions and after DA, GD and GD + DA treatments. Two-way ANOVA of total α-synuclein levels (f) revealed a significantly different effect (interaction: $F_{3-16} = 31.63$, $p < 0.0001$) of treatments ($F_{3-16} = 50.22$, $p < 0.0001$) upon control and differentiated ($F_{1-16} = 38.39$, $p < 0.0001$) SH-SY5Y cells. **+59.33% ($p < 0.001$) for GD-treated SH-SY5Y$^+$ cells and **+147.3% ($p < 0.001$) for GD + DA-treated SH-SY5Y$^+$ cells versus untreated SH-SY5Y$^+$ cells. GD + DA treatment significantly increased °+ 88% ($p < 0.001$) total α-synuclein levels with respect of GD treatment in differentiated cells. $^{\#}$+44.67% ($p < 0.01$) for GD-treated SH-SY5Y$^+$ cells versus DA-treated SH-SY5Y$^+$ cells. No statistically significant difference in total α-synuclein levels was observed after DA, GD and GD + DA treatments in control undifferentiated SH-SY5Y cells; $n > 10$ for each group.

GD + DA treatments. DA treatment did not induce any significant change in total α-syn content, thus indicating that DA is not able to affect α-syn synthesis in normal conditions but it could exacerbate its pathological changes after glucose starvation.

Interestingly, after GD and GD + DA treatments we observed only a 19 kDa immunopositive band corresponding to the full-length protein, with SYN-211 antibody (Fig. 6c) and two α-syn-immunopositive bands, one at 19 kDa, corresponding to the full-length protein, and the other at about 12 kDa corresponding to a carboxyl-terminal cleaved form, by using SYN-1 antibody (Fig. 6d). These observations indicate that GD, either alone or paired with DA overload, induces carboxyl-terminal α-syn cleavage in dopaminergic differentiated cells.

In line with these observations, densitometric analysis of α-syn immunopositive bands indicated a statistically significant increase of total α-syn levels in GD-treated SH-SY5Y$^+$ cells. This increase was higher SH-SY5Y$^+$ cells subjected to GD and DA treatment. GD, GD + DA and DA treatment did not modify DAT (Fig. 6a–b and g–h) and α-syn (Fig. 6c, d and i) levels in undifferentiated SH-SY5Y cells suggesting that differentiation into a dopaminergic neuron-like phenotype increases cell vulnerability to GD and DA treatment.

By IP coupled to WB techniques (Fig. 6e) we found that DAT co-immunoprecipitated with α-syn in untreated, DA-, GD- and GD + DA-treated SH-SY5Y$^+$ cells, indicating that DAT and α-syn directly interact and that the metabolic stress induced by GD + DA treatment does not affect their interaction.

GD-induced intracellular inclusions contain fibrillary aggregated insoluble α-syn

To characterize α-syn aggregates we performed thioflavin-S and α-syn double staining in SH-SY5Y$^+$ cells exposed to GD and DA overload. Thioflavin-S staining detects proteins that are aggregated into a fibrillary form. As shown in Fig. 7, thioflavin-S and α-syn double staining revealed that in SH-SY5Y$^+$ cells exposed to GD (Fig. 7g–i) and GD + DA (Fig. 7j–l), several α-syn-positive inclusions were also positive for thioflavin-S, indicating a fibrillar structure of the protein. Thioflavin-S-positive inclusions were not detected in untreated (Fig. 7a and b) or DA treated (Fig. 7d and e) SH-SY5Y$^+$ cells indicating that the fibrillar aggregation of α-syn occurred after the GD insult. Quantitative analysis of α-syn/thioflavin-S-positive inclusions at different time points after GD (Fig. 7m) revealed a progressive and statistically significant increase of α-syn/thioflavin-S-positive inclusions at 5, 15, 24 and 48 h after GD (statistical details are reported in the figure legend). This increase was remarkably higher when GD was paired with DA treatment, suggesting that high intracellular DA may foster the fibrillary aggregation of α-syn induced by GD.

Moreover, we performed sequential protein extraction with Triton X-100 and urea/SDS, to isolate the membrane bound α-syn fraction from the cytoplasmic insoluble fraction in both primary mesencephalic neurons and SH-SY5Y$^+$ cells. As reported in Fig. 8a sequential extraction of α-syn in primary mesencephalic neurons revealed that GD and GD + DA treatments induced the formation of high molecular weight species of α-syn in the Triton X-100-soluble fraction (lanes 2 and 3), corresponding to the membrane protein fraction, with respect to control cells where only monomeric α-syn was detected. High molecular weight species of α-syn were also present in the urea extracts, corresponding to reconstituted cytoplasmic insoluble protein fractions, from GD- and GD + DA-treated primary neurons (lanes 2 and 3). WB analysis (Fig. 8b) of sequential α-syn extracts also revealed that high molecular weight α-syn species were present in the TBS–Triton X-100 and urea fractions of GD + DA-treated SH-SY5Y$^+$ cells but not in undifferentiated SH-SY5Y cells. These findings indicate that GD and DA overload are able to induce α-syn aggregation in dopaminergic cells.

Inhibition of DA uptake and D_2R/D_3R stimulation counteract α-syn aggregation and cell injury induced by GD and DA overload

Our observations suggest that DA was able to exacerbate the neurotoxic changes brought about by GD in dopaminergic differentiated cells. In line with this, recent findings demonstrated that DA, L-DOPA and their quinone-metabolites may be toxic for dopaminergic neurons in metabolic stress conditions (Sulzer and Zecca 2000). Conversely, dopaminergic stimulation of D_2/D_3 autoreceptors has been found to exert neuroprotective effects (Ono *et al.* 2007). Therefore, we evaluated whether inhibition of DA uptake and D_2R/D_3R stimulation could prevent α-syn aggregation and neuron injury induced by GD + DA in SH-SY5Y$^+$ cells.

The data reported in Fig. 9a shows that cocaine treatment, that inhibited DAT function, exerted neuroprotective effects against GD + DA in SH-SY5Y$^+$ cells. Indeed, cell viability of SH-SY5Y$^+$ cells exposed to GD + DA in the presence of cocaine was comparable to that observed in untreated cells, suggesting that intracellular DA plays a central role in the neurodegenerative effects of GD. D_2R/D_3R stimulation by quinpirole was also able to prevent cell death induced by GD + DA. Moreover, when GD + DA-treated cells were incubated with both cocaine and quinpirole we observed that cell viability was increased in comparison with cocaine- or quinpirole-GD + DA-treated cells. Conversely, the D_2-like receptor antagonists L-sulpiride and spiperone exacerbated the neurodegenerative changes brought about by GD + DA treatment. Cocaine, quinpirole, spiperone and L-sulpiride did not affect cell viability of untreated SH-SY5Y$^+$ cells. These findings suggest that stimulation of D_2R/D_3R autoreceptors

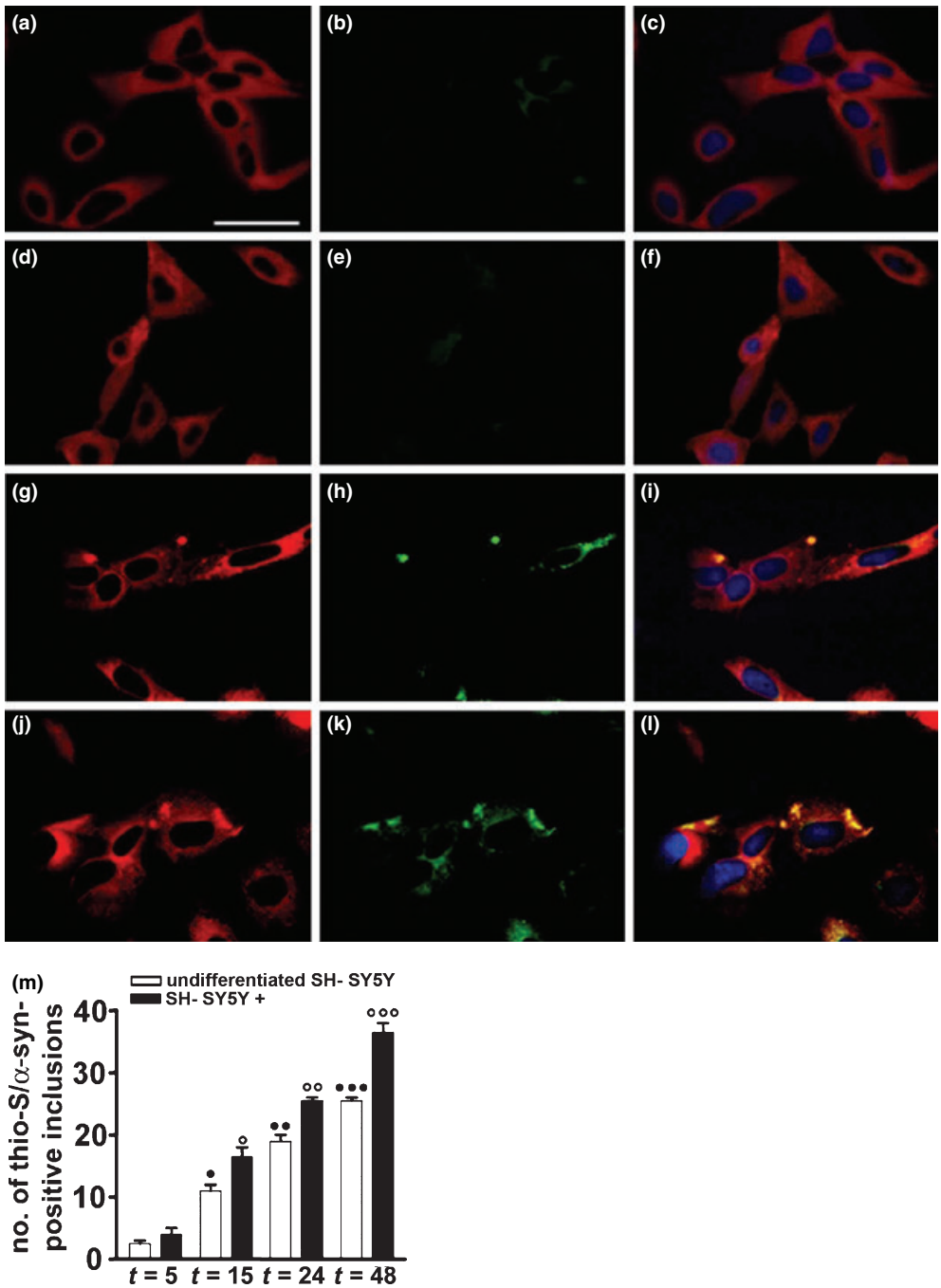


Fig. 7 (a–l) α-synuclein (α-syn; red) and thioflavin-S (green) double staining in SH-SY5Y$^+$ cells after GD and GD + DA treatments. Note the presence of thioflavin-S-positive α-syn inclusions (i–l) , indicative of the fibrillary structure of α-syn aggregates. Scale bars: 35 μm for (a–f). (m) Quantitative analysis of α-syn/thioflavin-S-positive inclusions in dopaminergic differentiated neuroblastoma cells after GD (white bars) and GD + DA (black bars) treatments. Two-way ANOVA revealed a statistically significant different effect of GD and GD + DA treatments (F_{1-6} = 18.47, $p < 0.01$) in inducing the increase in the number of α-syn-/thioflavin-S-positive inclusions at different time points ($F_{3,6}$ = 22.69, $p < 0.0001$) as indicated by the significant interaction ($F_{3,6}$ = 15.23, $p < 0.05$) between the two parameters (treatments and time points). Bonferroni's post-comparison test revealed that GD induced a statistically significant mean respective increase of 8.5 ($^{\bullet}p < 0.01$), 16.5 ($^{\bullet\bullet}p < 0.001$) and 23 ($^{\bullet\bullet\bullet}p < 0.001$) of the number of α-syn/thioflavin-S-positive inclusions at 15, 24 and 48 h from the insult with respect of the number of α-syn-/thioflavin-S-positive inclusions counted at 5 h from GD. The mean number of α-syn-/thioflavin-S-positive inclusions after GD + DA treatment was significantly increased at 15 (+12.5, $^{\circ}p < 0.001$), 24 (+21.5, $^{\circ\circ}p < 0.001$) and 48 h (+32.5, $^{\circ\circ\circ}p< 0.001$) from the insult.

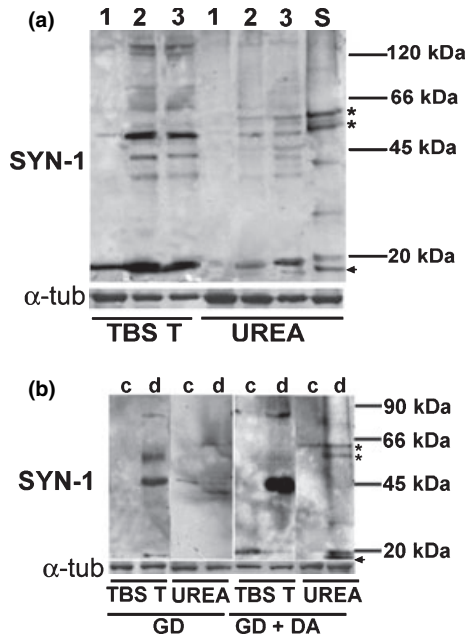


Fig. 8 (a) WB image of sequential α-synuclein protein extracts from untreated and GD- and GD + DA-treated mesencephalic cell cultures probed with SYN-1 antibody. Note the presence of high molecular weight α-synuclein bands in the TBS T-soluble fraction of GD- and GD + DA-treated cells. Cleaved (arrow) and high molecular weight (asterisks) species of α-synuclein, at the same high of those observed in dopaminergic differentiated neuroblastoma cells after GD + DA treatment (S) were also observed in the urea-soluble protein fraction of GD- and GD + DA-treated primary mesencephalic neurons. α-Tubulin bands are reported as loading controls. (b) Representative photomicrograph of a WB of sequential α-synuclein extracts from control (c) and SH-SY5Y$^+$ (d) cells after GD + DA treatment showing high molecular weight aggregates in the TBS T and urea-soluble fractions from GD- and GD + DA-treated differentiated cells. Note the presence of cleaved synuclein (arrow) and of two α-synuclein-immunopositive bands (asterisks) between 40 and 60 kDa in the urea-soluble fraction that are indicative of abnormal protein folding and aggregation. Please note that the band at 45 kDa is aspecific as previously reported (Perrin *et al.* 2003). α-Tubulin bands are reported as loading controls.

by DA is neuroprotective for dopaminergic neurons when the cells have been subjected to energy deprivation.

Figure 9b and c shows the effects of pharmacological treatments on DAT and α-syn levels in SH-SY5Y$^+$ cells. Densitometric analysis of DAT-immunopositive bands (Fig. 8b) revealed that cocaine treatment counteracted the decrease of DAT expression induced by GD paired with DA overload. Similarly, D_2R/D_3R stimulation by quinpirole also prevented the decrease of DAT expression. In GD + DA-treated cells, cocaine + quinpirole incubation further increased DAT levels in comparison with cocaine- or quinpirole-treated cells. Conversely, D_2R/D_3R blockade by L-sulpiride and spiperone exacerbated the decrease of DAT levels induced by GD + DA treatment.

Cocaine and quinpirole treatment also counteracted the increase of total α-syn levels induced by GD + DA (Fig. 9c), while spiperone and L-sulpiride further increased total α-syn content in GD + DA-treated cells. The effect of cocaine + quinpirole treatment on α-syn expression was additive, indeed in GD + DA-treated cells we observed that the incubation with both drugs further increased total α-syn levels in comparison with cocaine- or quinpirole-treated cells.

Furthermore, we observed that quinpirole (Fig. 10b) and cocaine (Fig. 10e) treatment also counteracted the formation of α-syn/DAT-positive inclusions induced by GD and DA overload in SH-SY5Y$^+$ cells (Fig. 10a). No intracellular inclusions were in fact detectable in cells exposed to GD and DA in the presence of quinpirole or cocaine, where α-syn and DAT stainings were particularly intense at the plasma membrane (arrows in Fig. 10b and e).

Spiperone and L-sulpiride treatments were rather able to exacerbate the formation of intracellular inclusions in GD + DA-treated cells. Indeed, as reported in Fig. 10, the intracellular α-syn-/DAT-positive inclusions were bigger and more frequent in spiperone (Fig. 10c) and L-sulpiride (Fig. 10d) treated cells that in cells exposed to GD and DA in the absence of D_2R antagonists.

Taken together, these findings suggest that intracellular DA is toxic to dopaminergic neurons in metabolic stress conditions that D_2R/D_3R autoreceptors are involved in the regulation of α-syn and DAT synthesis and trafficking and control α-syn aggregation in metabolic stress conditions, therefore modulating dopaminergic neuron resilience and vulnerability.

Discussion

The present results indicate that GD induces α-syn pathological changes such as carboxyl-terminal cleavage and aggregation into intracellular inclusions containing the DAT both in dopaminergic mesencephalic neurons and in SH-SY5Y cells differentiated into a dopaminergic-like phenotype. GD also decreased the total and membrane content of the DAT as well as the cell viability of dopaminergic cells, suggesting that glucose starvation may induce dysfunction and degeneration dopaminergic neurons. The formation of α-syn inclusions triggered by GD is exacerbated by intracellular DA uptake that also fosters DAT decrease and neuronal loss. The stimulation of D_2R/D_3R and the block of DA uptake prevent the formation of intracellular inclusions and exert neuroprotective effects.

Exhaustion of intracellular glucose after hypoglycaemia results in a reduction of mitochondrial membrane potential leading to decreased ATP generation and induces oxidative stress and reactive oxygen species production (Isaev *et al.* 2007) that in dopaminergic neurons contribute to the formation of DA-reactive metabolites, such as DA-quinones,

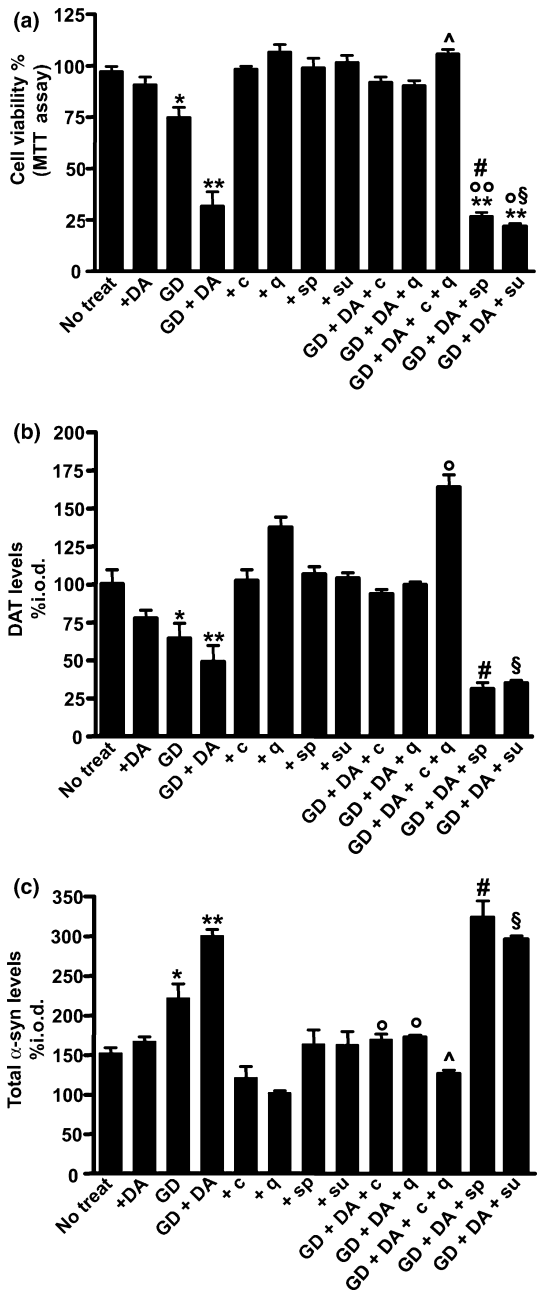


Fig. 9 Effects of cocaine (c), quinpirole (q), spiperone (sp) and L-sulpiride (su) treatments on cell viability and total DAT and α-synuclein expression levels of untreated and GD + DA-treated SH-SY5Y$^+$ cells. (a) Cell viability assay (MTT assay). Cell viability of SH-SY5Y$^+$ cells was significantly changed by the different drug treatments as revealed by the significant interaction of the ANOVA analysis ($F_{12,62} = 64.66$, $p < 0.0001$). Bonferroni's multiple comparison test revealed a respective significant reduction of *22.41% ($p < 0.01$) and 53.16% (**$p < 0.001$) in cell viability in GD- and GD + DA-treated cells with respect of untreated cells. Cell viability of cocaine- and quinpirole-GD+DA-treated cells was not different from that of untreated cells, indicating a neuroprotective effects of these drugs. Moreover in cocaine + quinpirole-GD + DA-treated cells, cell viability was increased with respect to cocaine- (^+13.67%, $p < 0.05$), quinpirole- (^+15.38%, $p < 0.05$) and GD + DA-treated cells. Spiperone and L-sulpiride treatments in GD + DA-treated cells brought about a significant reduction of 69.45% ($p < 0.001$) and 74.9% ($p < 0.001$) in cell viability in comparison with untreated cells. Moreover both treatments induced a significant reduction of 44.80% ($^{\circ\circ}p < 0.001$) and 18.1% ($^{\#}p < 0.01$) for spiperone and of 52.3% ($^{\circ}p < 0.01$) and 24.3% ($^{\S}p < 0.01$) for L-sulpiride in cell viability with respect to GD- and GD + DA-treated cells; $n > 10$ in each group. (b) Total DAT levels measured by western blotting coupled to densitometric analysis of bands. Mean DAT levels of SH-SY5Y$^+$ cells (Fig. 9b) after treatments were significantly different ($F_{12,48} = 10.04$, $p < 0.0001$) as indicated by one-way ANOVA. Bonferroni's post-comparison test showed that DAT levels were significantly decreased in GD- (*−35.93%, $p < 0.05$) and GD + DA-treated cells (**−51.13%, $p < 0.01$) with respect to untreated cells. In cocaine- and quinpirole-GD + DA treated cells, DAT levels were not reduced with respect to untreated cells. DAT levels of cocaine + quinpirole-GD + DA-treated cells were statistically increased ($^{\circ}$+ 63.57%, $p < 0.001$) with respect to untreated cells. Conversely, DAT levels in spiperone and L-sulpiride-GD + DA-treated cells were significantly decreased of −68.9 ($^{\#}p < 0.01$) and −65.15 ($^{\S}p < 0.01$), respectively, in comparison with untreated cells; $n > 10$ in each group. (c) Total α-synuclein levels measured by western blotting coupled to densitometric analysis of bands. One-way ANOVA showed that mean total α-synuclein levels (Fig. 9c) were significantly changed ($F_{12,38} = 40.45$, $p < 0.0001$) after treatments. GD- and GD + DA treatments induced a respective significant increase of 69.67% (*$p < 0.05$) and 148.3% (**$p < 0.001$) in α-synuclein levels in comparison with untreated cells. Cocaine and quinpirole incubation of GD + DA-treated cells showed no significant effect on total α-synuclein levels with respect to untreated cells. In GD + DA treated cells, cocaine + quinpirole treatment further reduced α-synuclein levels with respect to cocaine- (^+43%, $p < 0.05$) and quinpirole-incubated (^+45.87, $p < 0.05$) cells. Spiperone and L-sulpiride treatment induced a respective statistically significant increase of 174% ($^{\#}p < 0.001$) and 143.5% ($^{\S}p < 0.001$) of total α-synuclein levels in GD + DA-treated cells in comparison with untreated cells. In GD + DA -treated cells both treatments induced a significant increase of 102.3% ($^{\circ}p < 0.001$) for spiperone and 74.33% ($^{\circ}p < 0.01$) for L-sulpiride in total α-synuclein levels with respect to GD-treated cells; $n > 10$ in each group.

that ultimately lead to apoptosis (Sulzer and Zecca 2000; Emdadul *et al.* 2003). Recent '*in vitro*' evidences indicate that α-syn fibrillary aggregation can be exacerbated by its interaction with DA-quinones (Conway *et al.* 2001). In line with these evidences, our experiments showed that GD induced a significant increase of α-syn levels and the formation of fibrillary aggregated α-syn-immunopositive inclusions in SH-SY5Y$^+$ cells. Moreover, when GD was followed by DA treatment a further increase in α-syn levels and in fibrillary aggregated-α-syn-positive inclusions was observed. As α-syn plays a crucial role in the control of DA neuron function (Uversky 2007), these findings may indicate that glucose starvation could lead to PD-related cell dysfunctions through the formation of intracellular α-syn protein aggregates.

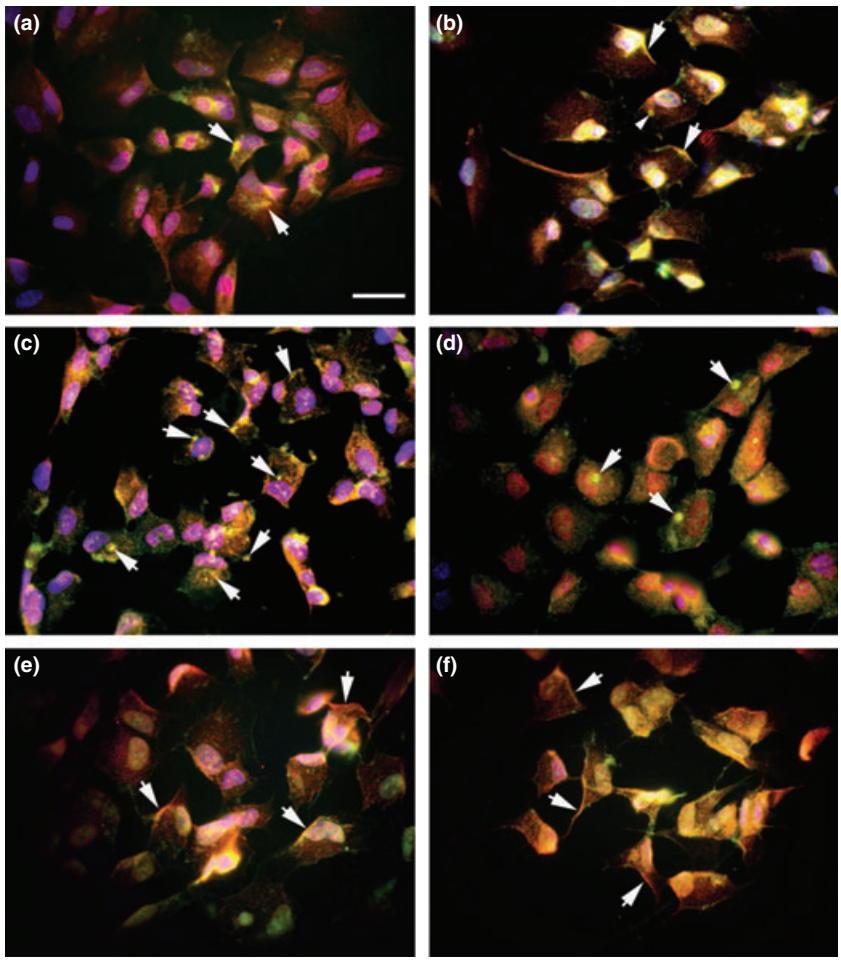


Fig. 10 Effect of drug treatments on the formation of DAT/α-synuclein-positive inclusion in GD + DA dopaminergic differentiated SH-SY5Y cells. Representative photomicrographs show the merge of α-synuclein (red) and DAT (green) staining in GD + DA treated dopaminergic differentiated SH-SY5Y cells (a) GD + DA treated SH-SY5Y$^+$ cells. Note the presence of intracellular α-synuclein/DAT-positive inclusions indicated by the arrows. (b) Representative photomicrograph showing the effect of quinpirole treatment on GD + DA treated SH-SY5Y$^+$ cells. Note the absence of intracellular inclusions and the presence of intense α-synuclein/DAT immunopositive staining at the plasma membrane (arrows). (c) Effect of Spiperone incubation on GD + DA treated SH-SY5Y$^+$ cells. Note the presence of big round-shaped α-synuclein/DAT-positive intracellular inclusions (arrows). (d) Representative photomicrograph showing the effect of L-sulpiride treatment on GD + DA-treated SH-SY5Y$^+$ cells inducing the formation of big round-shaped α-synuclein/DAT-positive inclusions (arrows) within cell cytoplasm. (e) Representative photomicrograph showing the absence of DAT- and α-synuclein-immunopositive inclusions within cocaine treated SH-SY5Y$^+$ cells exposed to GD + DA treatment. Please note the membrane distribution of DAT and α-synuclein as indicated by the arrows. (f) Effect of quinpirole + cocaine treatment of SH-SY5Y$^+$ cells exposed to GD + DA treatment. Please note the absence of DAT- and α-synuclein-immunopositive inclusions within the cells and the membrane distribution of DAT and α-synuclein (indicated by the arrows). Scale bars: 10 μm for (a–f).

Indeed, our results on rodent primary mesencephalic neurons showed that GD induces the formation of α-syn/DAT-positive inclusions within dopaminergic cells in parallel with an increase of total α-syn levels and with a decrease of total DAT levels. These effects were exacerbated by DA overload. These findings are reminiscent of postmortem studies showing that DAT mRNA levels were reduced in the SN of PD patients (Harrington *et al.* 1996; Counihan and Penney 1998) which is reach of LB during stages 3–4 of the disease (Braak *et al.* 2006). Interestingly, DA treatment differently regulated DAT and α-syn expression and trafficking in the primary cells not subjected to GD by increasing their colocalization at the plasma membrane. These results indicate that GD modulates both α-syn and DAT trafficking likely affecting the function of dopaminergic neurons by reducing DAT membrane content. Furthermore, our findings suggest that α-syn and DAT may share common trafficking mechanisms which are influenced by changes in energy support and by DA stimulation.

Overproduction of α-syn can lead to its aggregation and precipitation into intracytoplasmic aggregates (Uversky 2007). On this line, our data on primary mesencephalic neurons indicates that GD induces both an increase of α-syn levels and the formation of α-syn inclusions in dopaminergic cells. Furthermore, we observed that, in these inclusions, α-syn was associated with the DAT, whose levels were decreased by GD. DA overload brought about a further increase in α-syn levels and a major decrease of DAT content in dopaminergic cells.

By using human SH-SY5Y$^+$ cells, we showed that GD reduces cell viability/mitochondrial function of dopaminergic cells. In agreement with what we observed in primary mesencephalic neurons, GD increased total α-syn content also in these cells. The western blot studies showed that GD induced other pathological changes in α-syn such as its carboxy-terminal cleavage and the formation of high molecular weight aggregates both in the membrane and cytoplasmic fractions of SH-SY5Y$^+$ cells and mesencephalic neurons in primary culture. Low molecular weight α-syn species are present in LB (Uversky 2007) and human α-syn lacking the 20 carboxy-terminal amino acids assembles into filaments '*in vitro*' faster than either wild-type or mutant protein (Tofaris and Spillantini 2005). Therefore, the GD-induced α-syn cleavage may have further exacerbated the aggregation of the protein into insoluble fibrils. Indeed, in SH-SY5Y$^+$ cells numerous α-syn inclusions were also thioflavin-S-positive, indicating a fibrillary state of the protein. All the observed GD-induced alterations, were enhanced by DA treatment. In particular, glucose-deprived dopaminergic cells exposed to DA showed increased number of thioflavin-S-/α-syn-positive inclusions indicating that DA increases α-syn fibrillary aggregation.

Like in mouse primary dopaminergic neurons, DAT levels were decreased also in SH-SY5Y$^+$ cells but not in control undifferentiated neuroblastoma cells. Our immunocytochemical studies indicated that DAT and α-syn subcellular localization were affected by GD also in SH-SY5Y$^+$ cells. Indeed, DA treatment stimulated DAT and α-syn membrane translocation, while after GD we observed the intracellular precipitation of the proteins to form intracytoplasmic inclusions. The formation of α-syn-/DAT-positive inclusions, induced by GD, coincided with the decrease of DAT levels at the plasma membrane observed by WB that was exacerbated by DA treatment. These findings further indicate that α-syn and DAT share common and equally regulated trafficking systems that can be modulated by nutrient deprivation suggesting the occurrence of an interaction between these two proteins. DA stimulation can also modulate the trafficking of the two proteins but its effect is different in the normal or in the glucose deprived cells. Moreover, our co-IP experiments showed that DAT interacts with α-syn and that this interaction is not affected by the neurotoxic stimuli. All these observations are consistent with previous studies describing a fine regulation of DAT trafficking and function by its interaction with α-syn in normal and oxidative stress conditions (Lee *et al.* 2001; Wersinger *et al.* 2003).

Our findings suggest that α-syn aggregation may participate to DA neuron dysfunction by inducing a decrease of DAT membrane content as the latter may dissociate from the membrane and precipitate into intracytoplasmic inclusions together with misfolded α-syn. An increased intracellular DA uptake after GD may exacerbate these phenomena because it increases the levels and the fibrillary aggregation of α-syn while it decreases DAT membrane content. These observations are reminiscent of recent PET studies indicating that L-DOPA treatment further decreases striatal DAT membrane content in the brain of PD patients (Fahn 2005).

To evaluate whether DA increases the GD-induced α-syn aggregation by acting at the intracellular level or by stimulating D_2R/D_3R we treated the SH-SY5Y$^+$ cells either with the DA-uptake blocker cocaine or with the D_2R/D_3R agonist quinpirole. Cocaine treatment exerted neuroprotective effects by counteracting the GD + DA-induced decrease of both cell viability and DAT levels and the increase of total α-syn levels. Moreover, inhibition of DA uptake prevented the formation of α-syn-/DAT-positive inclusions. Looking at these findings it is feasible to speculate that cocaine may have counteracted the GD + DA-induced cell death and α-syn/DAT aggregation acting at least through three different mechanisms (1) by blocking DA uptake and the consequent formation of DA-toxic metabolites that can exacerbate α-syn aggregation; (2) by inducing an increase of extracellular DA that may result in a higher stimulation of D_2R/D_3R whose activation can enhance DAT (Bolan *et al.* 2007) and α-syn membrane translocation; and (3) by directly stimulating the membrane translocation of both the DAT and α-syn. In line with these hypothesis, recent observations (Qin *et al.* 2005) indicate that increased α-syn and DAT membrane translocation occur in the striatum of cocaine abusers, indicating that DAT blockers can modulate the trafficking of both proteins and that α-syn may play a role in the regulation of DA synaptic tone.

Our results also indicate that the stimulation of GD + DA-treated SH-SY5Y$^+$ cells by the D_2R/D_3R agonist quinpirole exerts neuroprotective effects that are perfectly comparable to those observed with cocaine treatment. Indeed, quinpirole counteracted the GD + DA-induced cell death, the DAT decrease and the α-syn increase. Furthermore, it reduced the formation of α-syn-/DAT-positive inclusions. Conversely, the D_2-like receptor antagonists L-sulpiride and spiperone worsened the noxious effects of GD + DA overload, by further decreasing cell viability and DAT levels and by increasing α-syn expression and the formation of α-syn-/DAT-positive inclusions in SH-SY5Y$^+$ cells. It has been previously reported that pre-incubation with D_2R-like agonists provides neuroprotection against toxicity induced by calcium overload and

exposure to superoxide anions, as the activation of D_2R can induce the synthesis of proteins that scavenge free radicals (Sawada *et al.* 1998). The stimulation of D_2R by quinpirole could have counteracted the GD + DA-induced neurodegeneration also in our model, by reducing the GD-related oxidative insult and consequently by lowering DA toxicity. Furthermore, stimulation of D_2R is able to increase the surface expression of the DAT through a direct D_2R/DAT interaction (Bolan *et al.* 2007; Lee *et al.* 2007). The latter findings suggest that the stimulation D_2R/D_3R by quinpirole could have been able to increase the membrane expression of the DAT, therefore counteracting the intracellular translocation of α-syn/DAT complexes induced by GD + DA-treatment.

Our results also indicate that cocaine and quinpirole effects on the cell viability as well as on DAT and α-syn expression levels in SH-SY5Y$^+$ cells exposed to GD were additive. Moreover, in cocaine + quinpirole-treated cells, DAT/α-syn inclusions were absent; indeed, the proteins were distributed to the plasma membrane. These observations suggest that the effect of these two drugs on the formation of DAT/α-syn inclusions is additive and that cocaine may have supported the neuroprotective effect of D_2R/D_3R stimulation by increasing extracellular DA levels.

Taken together, our findings indicate that reduced glucose support can induce parkinsonian-like α-syn changes as well as DAT-related dopaminergic neuronal dysfunctions. The aggregation of α-syn and DAT into intracellular inclusions induced by GD may be directly implicated in the induction of the dysfunctions of dopaminergic cells as it is associated to a decrease in the membrane translocation of the DAT. DA overload exacerbates the GD-induced α-syn and DAT pathological changes, suggesting that L-DOPA treatment of PD patients may foster α-syn-related pathological alterations as well as dopaminergic neuron dysfunctions involving the DAT. Our observations also indicate that D_2R/D_3R agonists treatment and DA-uptake inhibitors exert neuroprotective effects as these drugs can counteract the pathological alterations brought about by GD + DA treatment. Indeed, D_2R/D_3R stimulation reduced cell death and counteracted the increase in α-syn levels and the decrease of DAT expression. Moreover, these compounds prevent the formation of GD + DA-induced intracellular α-syn-/DAT-positive inclusions.

The results of this study are relevant for a better understanding of the molecular mechanisms involved in PD pathogenesis and may represent a powerful tool to identify novel pharmacological targets to revisit and redesign therapeutic strategies to cure the α-syn-related DA neuron dysfunctions.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1

Fig. S2 TH and α-synuclein double staining in primary mesencephalic neurons in basal conditions (A–C) after GD (D–F), GD + DA (G–I) and DA treatment (J–L).

Fig. S3 Thioflavin-S single staining in SH-SY5Y+ cells.

Fig. S4 Western blotting showing control immunostaining with an irrelevant antibody (GFAP) on the gel containing the immunoprecipitates of fig.

Appendix S1 Supplementary information.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2008.05406.x (This link will take you to the article abstract).

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