Autophagy enhancement is rendered ineffective in presence of α -synuclein in melanoma cells

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Abstract Increased cellular concentration of α -synuclein (α -syn) predisposes it to misfolding and aggregation that in turn impair the degradation pathways. This poses a limitation to the use of overexpression models for studies on α -syn clearance by autophagy, which is widely investigated for its therapeutic potential. This limitation can be overcome with the use of endogenous models. In this study, SK-MEL-28, a melanoma cell model with endogenous α -syn expression, was employed to study α -syn clearance through autophagy. We demonstrated the dual localization of α -syn to nucleus and cytoplasm that varied in response to changes in cellular environment. Autophagy inhibition and exposure to dopamine favored cytoplasmic localization of α -syn, while autophagy induction favored increased localization to the nucleus. The inhibitory effect of dopamine on autophagy was heightened in presence of α -syn. Additionally, because α -syn had a regulatory effect on autophagy, cells showed an increased resistance to autophagy induction in presence of α -syn. This resistance prevented effective induction of autophagy even under conditions of prolonged autophagy inhibition. These results highlight alternate physiological roles of α -syn, particularly in non-neuronal cells. Because autophagy enhancement could reverse neither the increase in α -syn levels nor the autophagy

inhibition, there arises a need to evaluate the efficacy of autophagy-based therapeutic strategies.

Keywords Autophagy · Melanoma · Parkinson's disease · α -Synuclein

Introduction

α -Synuclein (α -syn) is a 14 kDa protein, found in the brain, that plays a regulatory role in synaptic transmission (Stefanis 2012). Natively, it occurs as an unfolded protein grouped under the family of intrinsically disordered proteins and exhibits flexibility in its folding states. Due to lack of a defined secondary structure, α -syn is capable of attaining both the alpha helix and the beta sheet under different cellular conditions (Uversky 2008). Misfolding and aggregation of α -syn is observed in a diverse group of neurodegenerative diseases, such as Parkinson's disease (PD) and multiple system atrophy, that are broadly classified as synucleinopathies (Uversky 2008). During the course of aggregation, α -syn can self-associate forming intermediates of various sizes such as dimers, trimers, and oligomers that eventually form the large aggregates visualized as inclusion bodies seen in synucleinopathies (Uversky 2008). Because misfolding of proteins is central to pathogenesis of neurodegenerative diseases, factors that contribute to misfolding have been a subject of great interest. In this regard, it has been observed that increased cellular concentration of the protein in itself can act as a trigger for misfolding, and direct evidence for this comes from familial PD harboring multiplications of *SNCA* (Kim and Lee 2008). Cells exert control at both the transcriptional and translational levels to

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prevent protein accumulation; therefore, accumulation of proteins would be a result of failure in protein clearance pathways, particularly in absence of genetic changes.

The cells employ two major pathways for protein clearance—Ubiquitin-Proteasome pathway (UPS) and Autophagy-lysosomal pathway (ALP). UPS is used to clear ubiquitin-tagged proteins (Lecker et al. 2006), while ALP functions as a bulk degradation pathway that clears misfolded proteins and damaged organelles in turn contributing to cellular homeostasis (Glick et al. 2010). This makes ALP the preferred target for treatment strategies (Rubinsztein 2006). With a view to understanding degradation kinetics, the clearance of α -syn has been widely studied with both the UPS and ALP being implicated (Webb et al. 2003; Vogiatzi et al. 2008; Ebrahimi-Fakhari et al. 2011). Misfolded α -syn impairs degradation pathways blocking degradation of itself and other target substrates (Xilouri et al. 2009; Emmanouilidou et al. 2010). Additionally, overexpression of α -syn has also been shown to block macroautophagy (Winslow et al. 2010). A common theme in neurodegenerative diseases is the saturation and impairment of degradation pathways by misfolded proteins that fuels further accumulation of cellular proteins and damaged organelles leading to cytotoxicity (Martinez-Vicente and Cuervo 2007). The wide utilization of overexpression models for studying α -syn clearance results in conflicting observations because the underlying protein burden in the cells not only predisposes α -syn to misfolding, but may also result in impairment of degradation pathways. Therefore, we hypothesize that models with endogenous α -syn may be better suited to study degradation of α -syn and will enable us to overcome the shortcomings of overexpression models, where the protein concentration may favor both misfolding and impairment of degradation pathways.

Although conventionally believed to be a neuronal protein, recent reports have demonstrated the presence of α -syn in non-neuronal cells such as red blood cells, T-lymphocytes, and melanoma where its physiological relevance remains unknown (Barbour et al. 2008; Matsuo and Kamitani 2010; Colasanti et al. 2014). Given the close parallels between melanin and dopamine synthesis (Pan et al. 2011), we employed the melanoma model, SK-MEL-28, that expresses α -syn endogenously to evaluate degradation of α -syn through ALP. The effect of dopamine addition on α -syn behavior in these cells was also studied. Our results showed the dual localization of α -syn to both the nucleus and cytoplasm. This distribution was affected by changes in autophagic flux and presence of dopamine. Furthermore, we observed that α -syn appeared to exert

a regulatory effect on autophagy and this regulatory effect interfered with enhancement of autophagy by chemical means, even under conditions when autophagy was inhibited in the cells.

Materials and methods

Materials

All reagents were purchased from Sigma-Aldrich (Bengaluru, India), unless specified otherwise. Torin-1 was purchased from Tocris Bioscience (Bristol, UK) and Chloroquine was a kind gift from the Department of Biochemistry (SCTIMST, Trivandrum, India). All antibodies were purchased from Abcam (USA) and anti-Histone antibody was a kind gift from Rajiv Gandhi Centre for Biotechnology (RGCB, Trivandrum, India).

Cell culture

The cell line SK-MEL-28 was purchased from the cell repository at RGCB, Trivandrum, India. Cells were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics at 5% CO₂ at 37 °C.

Cell viability

Cell Viability was measured by adding the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to cells at a concentration of 1 mg/mL diluted in Phosphate Buffered Saline and incubated for 2 h at room temperature in the dark. The resulting formazon crystals were dissolved in acidified isopropanol and absorbance was measured at 575 nm with 630 nm as reference wavelength on a microplate reader (Tecan, Switzerland). The viability of the treated cells was expressed as the percentage of the test absorbance divided by the control absorbance (Mosmann 1983).

Flow cytometry

The cells were processed as per the protocol recommended by the antibody manufacturer (Abcam). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton-X 100 and blocked with 5% fetal bovine serum. The cells were then incubated with anti- α -syn (1: 100) for 2 h at 4 °C, washed and probed with FITC-tagged secondary antibody (1:100) and fluorescence captured on BD FACS Aria.

Cell fractionation

The fractionation was achieved by using the previously published protocol (Suzuki et al. 2010). The nuclear, cytoplasmic and whole cell fractions were then subject to immunoblotting.

Immunoblotting

Standard western blotting protocol was followed (Mahmood and Yang 2012). Cells were lysed in ice-cold RIPA buffer by incubating in ice for 30 min and centrifuged at 16,000g for 15 min. Supernatant was collected and protein estimated by the Bradford method (Bradford 1976). 30 μ g protein was loaded onto gels (10% or 15%) and transferred onto PVDF membrane. The membranes were blocked with 5% skimmed milk in Tris-Buffered Saline containing 0.5% Tween-20 (TBST) and incubated overnight with primary antibody (anti- α -syn [1:30,000], anti-LC3 [1:1000], anti-p62 [1:50,000], anti-beta actin [1:2500], anti-Histone [1:500]). Membranes were then probed with HRP-tagged secondary antibody (1:10,000 or 1:30,000), bands detected using Chemiluminescence (Millipore) and captured on X-ray films. The X-rays films were imaged using Uvipro Platina (UVItect Gel documentation system) and densitometry analysis was performed using ImageJ analysis software. The bands were quantified and normalized with their respective β -actin.

Immunostaining

The immunostaining was done using previously published protocol with some modifications (Winslow et al. 2010). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton-X 100 and blocked in 5% fetal bovine serum. The cells were incubated with anti- α -syn (1:100) overnight and then probed with a FITC-tagged secondary antibody (1:200). The cells were counterstained with 5 μ g Hoechst stain and imaged on fluorescence microscope Olympus IX51 with Rolera XR (QImaging) camera at 40 $\times$ magnification. The images were obtained in grey scale and pseudo colored using the ImageJ software. All images were taken at room temperature.

RNA extraction and semi-quantitative PCR

The cells were harvested in TRI Reagent and RNA was extracted as per manufacturer's instructions. cDNA was synthesized from 2.5 μ g RNA using Verso cDNA synthesis kit (ThermoFisher Scientific). Semi-quantitative PCR was carried out with 2 μ L cDNA (GeneAmp PCR System 9700 [Applied Biosystems]) using DNase Taq DNA Polymerase (ThermoFisher Scientific). Reaction conditions were as follows: 95 $^{\circ}$ C

for initial denaturation and 35 cycles of 95 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s and a final extension at 72 $^{\circ}$ C for 5 min. The PCR amplified products were run on 1% agarose gel, imaged on Uvipro Platina (UVItect Gel documentation system), and analyzed using ImageJ software. The bands were quantified and normalized with their respective β -actin. Previously reported primers were used and purchased from Sigma (McLean et al. 2012).

α -synuclein – Forward- 5'-AAA ACC AAG GAG GGA GTG GT

Reverse - 5'-GCC TCA TTG TCA GGA TCC AC

β -actin – Forward- 5' -CATGTACGTTGCTATCCAGGC

Reverse - 5'- CTCCTTAATGTCACGCACGAT

Transfection

The siRNA duplex targeting α -syn was designed and purchased from Eurogentech (SNCA-S GGU-GUU-CUC-UAU-GUA-GGC-U55 SNCA-AS AGC-CUA-CAU-AGA-GAA-CAC-C55) as a lyophilized powder that was reconstituted in RNase free water. A final concentration of 1 nM of the siRNA duplex diluted in OptiMEM (Invitrogen) was used for transfection using the INTERFERin® (Polyplus Transfection) transfection reagent as per manufacturer's instructions. (Nilsson et al. 2014). All treatments were carried out 48 h post transfection and cells were harvested in TRI reagent. RNA and protein were extracted and used for semi-quantitative PCR and immunoblotting as described earlier.

Exogenous dopamine uptake

Dansyl-Dopamine synthesized as described previously (Oberman et al. 1970) was a kind gift from FADDS Laboratory, SCTIMST, Trivandrum. Cells were treated with varying concentrations of Dansyl-Dopamine in duplicates and incubated for 8 h at 37 $^{\circ}$ C, following which the cells were fixed with 4% paraformaldehyde and stained with 5 μ g Hoechst. The cells were imaged under a fluorescence microscope Olympus IX51 with Rolera XR (QImaging) camera and pseudo colored using ImageJ software.

Thiobarbituric acid Reactive Substances (TBARS) assay

TBARS assay was carried out as described previously (Buege and Aust 1978). Cells were lysed in 200 μ L of

Fig. 1 Distribution of α -syn. **a** The flow cytometry profile of SK-MEL-28 cells stained for α -syn probed with FITC conjugated secondary antibody showing a clear shift in the histogram of cells stained for α -syn (marked as test) when compared with cells exposed only to FITC conjugated secondary antibody (marked as secondary only) confirming that SK-MEL-28 cells express α -syn. **b** Immunostaining of SK-MEL-28 cells for α -syn probed with FITC conjugated secondary antibody and counter stained with Hoechst reveals the nuclear and cytoplasmic localization of α -syn. Inset shows enlarged image of a single cell. (Scale bar = 50 μ m). **c** Immunoblots of the nuclear and cytoplasmic fraction with differing band patterns of α -syn with the nuclear fraction (NF) having a band corresponding to a molecular weight of 18 kDa (visualized as a single lower peak in the densitometry analysis) and the cytoplasmic fraction (CF) having a band corresponding to a molecular weight of 32 kDa (visualized as a single upper peak in the densitometry analysis) while the whole cell lysate (WCL) shows both bands (visualized as two peaks upper and lower in the densitometry analysis). The 18 and 32 kDa bands were the only predominant bands even when the blots were overexposed

5% SDS and boiled in the presence of 1 mL of 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl containing solution until a pink color was obtained. Supernatant was collected and absorbance measured at 535 nm on a UV spectrophotometer. Distilled water boiled with the reagent was used as blank. The formula $(\text{Test O.D} \times \text{Total volume}) / (0.000156 \times \text{volume taken} \times 1000)$ was applied and the levels of MDA expressed in nmol/mg of total protein.

Melanin assay

The melanin content was assayed by a modified protocol as described previously (Pan et al. 2012). Cells were harvested by trypsinization, re-suspended in 1 mL

culture medium and counted on a hemocytometer. The cells were then pelleted down by centrifugation and boiled with 200 μ L 1 N NaOH at 80 $^{\circ}$ C for 1 h. The supernatant was collected by centrifugation at 1500 rpm for 5 min and the absorbance at 415 nm was measured using microplate reader (Tecan, Switzerland). Serial dilution of melanin (1 mg/mL) was used to prepare standard curve. Melanin content for each sample was normalized with the number of cells and expressed as amount in 10,000 cells.

Statistical analysis

Statistical analysis was carried out using Graph Pad Prism software (Version 6). Statistical significance was

Fig. 2 Autophagy induction. **a** Western blots and the corresponding graphs showing an increase in LC3-II and a decrease in p62 after treatment with 2 nM Torin-1 (T2) and 10 nM Torin-1 (T10) for 12 h. **b** Western blot and graph showing an increase in α -syn after treatment with Torin-1 at 2 nM (T2) and 10 nM (T10) concentration for 12 h ($n = 3$, $*p < 0.05$, compared to control). **c** Increase in mRNA expression of α -syn after treatment with Torin-1 at 10 nM concentration from 6 h assayed through semi-quantitative PCR ($n = 3$, $*p < 0.05$) where T2 is Torin-1 treatment at 2 nM and T10 is Torin-1 treatment at 10 nM. **d** The cell viability upon treatment Torin-1 was measured by the MTT assay.

analyzed using Student's *t*-test and One-way ANOVA, and a *p* value < 0.05 was considered significant.

Results

Alterations in autophagy affect α -syn expression and sub-cellular distribution Although the presence of α -syn in SK-MEL-28 has been reported previously (Matsuo and Kamitani 2010), we confirmed its presence through flow cytometry and immunostaining. Results showed 97% of the cells were positive for α -syn (Fig. 1a), and it was found to be localized to both the nucleus and cytoplasm (Fig. 1b). To further verify its nuclear and cytoplasmic distribution, these fractions were subjected to immunoblotting. In addition to the

Torin-1 was dissolved in DMSO, which was also included as a vehicle control. There was no loss in viability with DMSO alone or at 2 nM. There was a very slight reduction in cell viability at 10 nM, which was not significant. ($n = 3$, $^{ns}p > 0.05$) (V10 represents the volume of vehicle corresponding to 10 nM Torin-1, T2 represents 2 nM, and T10 represents 10 nM Torin-1 treatment respectively). **e** Western Blot and graphs showing changes in α -syn and LC3 / p62 respectively, following serum starvation for 24 h where w/o S represents without serum condition ($n = 3$, $*p < 0.05$)

dual localization of α -syn, we also observed that the monomeric α -syn corresponding to 18 kDa was confined to the nucleus while a higher molecular weight species corresponding to 32 kDa appeared exclusively in the cytoplasm, which conspicuously lacked in the presence of the monomeric α -syn (Fig. 1c). The EP1646Y anti- α -syn antibody used here has been previously shown to be capable of detecting higher molecular weight species (Roostae et al. 2013).

Because α -syn is known to occur physiologically as a monomer, we evaluated the effect of autophagic alterations on the expression and distribution of monomeric α -syn despite its nuclear localization. Initially, autophagy was induced to test if it resulted in enhanced clearance of α -syn by treating the cells with autophagy inducer Torin-1, an mTOR inhibitor (Thoreen et al. 2009).

Fig. 3 Autophagy inhibition. **a** Western Blots and their corresponding graphs showing increase in autophagy markers LC3-II and p62, after treatment with two different concentrations of Chloroquine at 10 μ M (C10) and 20 μ M (C20) for 12 h. **b** Western blot image and graph showing a modest increase in α -syn levels after treatment with two different concentrations of Chloroquine at 10 μ M (C10) and 20 μ M (C20) for 12 h. ($n = 3$, $*p < 0.05$, $^{ns}p > 0.05$, compared to control)

LC3-II is correlated with the number of autophagosomes and serves as an indicator of autophagosome formation, while also being degraded by autophagy. Most antibodies for LC3 have a higher sensitivity for LC3-II. For these reasons, LC3-II levels were compared between samples to determine the changes in autophagic flux (Mizushima and Yoshimori 2007). Torin-1's effect on autophagy was confirmed by assaying for changes in LC3-II and p62, which showed an approximately 2-

fold increase and 2-fold decrease, respectively (Fig. 2a). Contrary to expectation, we found a significant 4-fold increase in α -syn levels when autophagy was induced (Fig. 2b). Interestingly, this increase in autophagy did not lead to an increase in α -syn in the cytoplasm; instead, stronger signals were obtained from the nucleus as observed with immunostaining (Fig. 8, panel A). The increase in protein level was also reflected in the mRNA level, which showed an approximately 1.5-fold

Fig. 4 Autophagy induction with knockdown of α -syn. **a** The viability of α -syn knockdown cells after treatment with 10 nM Torin-1 (T10 K) for 8 h was compared with Torin-1 treated cells with intact α -syn expression (T10) and a 50% loss in viability was observed in the knockdown cells ($n = 3$, $*p < 0.05$). **(b)** Western Blot and graph showing a decrease in p62 in α -syn knockdown cells (T10 K) when treated with 10 nM Torin-1 for 8 h when compared with Torin-1 treated cells with intact α -syn expression (T10)

increase within 6 h after the addition of the drug (Fig. 2c). We then tested to see if α -syn showed a similar response under serum starvation, which is an alternate mechanism to induce autophagy (Li et al. 2013). Although Torin-1 treatment did not result in any loss of viability (Fig. 2d) serum starvation resulted in loss of cell viability, yet the surviving cells showed a significant 2.5-fold increase in α -syn levels (Fig. 2e). These results suggest that autophagy induction enhances α -syn expression at both transcriptional and translational levels and may be essential for cell survival.

Since the induction experiments did not result in increased clearance of α -syn, conversely, we followed it up with autophagy inhibition to determine if it caused accumulation of α -syn. The inhibition of autophagy was achieved through chemical inhibitor chloroquine

(Solomon and Lee 2009). Its inhibitory effect was confirmed by assaying for LC3-II and p62 levels, which showed a 7.5-fold and 4-fold, increase respectively at 20 μ M concentration, as opposed to the decrease in p62 levels observed with Torin-1 treatment (Fig. 3a). The 7-fold increase in LC3-II compared to the 2-fold increase with Torin-1 also suggests that the increase in LC3-II observed with chloroquine is due to LC3-II accumulation, resulting from lack of degradation. The inhibition of autophagy led to a small but insignificant increase in α -syn levels (Fig. 3b). Despite the minimal effect on monomeric α -syn levels, we observed an increase in α -syn in the cytoplasm as observed with immunostaining, when autophagy was inhibited (Fig. 8, panel B). This suggests that although autophagy may not mediate α -syn clearance, its inhibition affects subcellular distribution of α -syn by favoring its trafficking and retention in the cytoplasm.

Knockdown of α -syn makes cells sensitive to autophagy induction The two conditions of autophagy induction through Torin-1 and serum starvation elicited varied response in terms of viability, but both resulted in increase in monomeric α -syn protein levels, while Torin-1 also resulted in increased mRNA levels. This led us to postulate that α -syn may exert control over the extent of autophagy induction. To verify this, α -syn expression was knocked down using RNA interference and these cells were treated with autophagy inducer, Torin-1. Cells with α -syn knockdown (extent of knockdown, 85%; data not shown) showed a loss in viability when treated with Torin-1 although the knock-down itself did not result in any loss in viability (Fig. 4a). These results were in agreement with our earlier observations with serum deprivation and indicate that critical levels of α -syn are necessary for cell survival. Besides, the 2-fold decrease in p62 levels observed within the first 8 h (Fig. 4b) compared to that of the 12-h time point in cells with normal levels of α -syn indicated that the onset of autophagy induction happens at an earlier time point in the absence of the protein. These results confirm that a) presence of α -syn is essential for cell survival and b) α -syn has a regulatory role in autophagy pathway.

Exogenous dopamine addition irreversibly inhibits autophagy in SK-MEL-28 and autophagy induction in these conditions causes cell death The preferential loss of dopaminergic neurons in PD is attributed partly to the presence of dopamine itself. In addition to the predominant oxidative stress generated by dopamine metabolism and the conformational changes of α -syn induced in presence of dopamine, intermediates of dopamine metabolism can inhibit autophagy (Outeiro et al. 2009; Segura-Aguilar et al. 2014). Dopamine mediated autophagy inhibition could be a likely factor leading to failure in autophagy in dopaminergic cells.

Fig. 5 Effect of exogenous dopamine on cell viability. **a** Cell viability after addition of dopamine was measured using the MTT assay, and it was found that SK-MEL-28 cells did not show any loss of viability upto 1 mM concentration but a more than 50% loss in viability was observed at 3 mM ($n = 3$, $*p < 0.05$, compared to control). **b** The TBARS assay was used to measure the content of malondialdehyde (expressed as nmoles/mg of total protein) in the cells as a measure of ROS generation after addition of

1 mM dopamine for 24 h and it was found that dopamine addition did not lead to a significant increase in ROS compared to untreated controls ($n = 3$, $^{ns}p > 0.05$). **c** Dopamine tagged to dansyl chloride was added to SK MEL 28 cells at concentrations of 0.75, 1, and 3 mM and green fluorescence observed after 8 h. SK MEL 28 cells showed dopamine uptake at all three concentrations. (Scale Bar = 50 μ m)

We examined the effect of exogenous dopamine as another cellular parameter that could influence autophagy in SK-MEL-28. As a first measure, dopamine toxicity was tested. Despite the reportedly high toxicity of dopamine in vitro, SK-MEL-28 cells showed relatively high resistance to dopamine up to 1 mM (Fig. 5a). Interestingly, no significant production of reactive oxygen species was observed compared to untreated controls (Fig. 5b). However, this resistance to exogenous dopamine was not a result of lack of uptake as dopamine uptake was found to be highly efficient in these cells (Fig. 5c). We then proceeded to check if dopamine affected autophagy and found that it triggered inhibition of autophagy within 8 h and was sustained up to 24 h (Fig. 6a, b). Although addition of Torin-1 to dopamine treated cells minimized the initial onset of autophagy inhibition, it could neither prevent nor reverse dopamine induced autophagy inhibition over a period of 24 h (Fig. 6a, b). The combined treatment of Torin-1 and dopamine also resulted in loss of

viability over 24 h although neither of them individually induced cell death (Figs. 6c, 5a, 2d).

Dopamine causes an increase in α -syn levels and its effect on autophagy inhibition is mediated via α -syn

Given that dopamine is known to induce conformational changes in α -syn (Outeiro et al. 2009), its effect on basal expression of monomeric α -syn was investigated. Dopamine addition resulted in a 4-fold increase in α -syn protein levels within the first 8 h of dopamine addition and was sustained up to 24 h (Fig. 7a) and was accompanied with increased cytoplasmic localization of α -syn (Fig. 8, panel c). As observed with dopamine induced autophagy inhibition, addition of Torin-1 had no effect on clearance of excess α -syn induced in presence of dopamine (Fig. 7a). Since Torin-1 addition reversed neither dopamine induced autophagy inhibition nor the increase in α -syn levels, we tested to check if dopamine induced autophagy inhibition was mediated by α -syn. Dopamine was added to cells in which α -

Fig. 6 Effect of exogenous dopamine on autophagy. **a** and **(b)** Western Blot and graphs showing changes in autophagic markers p62 and LC3-II after treatment with 1 mM dopamine alone and dopamine with 10 nM Torin-1 (T10D) for two time points of 8 and 24 h respectively ($n = 3$, * $p < 0.05$, compared to control unless specified). **c** Cell viability,

measured by MTT assay, was compared between cells with combined treatment of 1 mM dopamine and 10 nM Torin-1 (T10D) and dopamine alone treated cells for 24 h at which dopamine and Torin-1 combined treatments (T10D) led to a 50% loss in viability ($n = 3$, * $p < 0.05$)

syn expression was knocked down and an approximately 2-fold decrease in p62 levels was observed when compared to dopamine treated cells with intact α -syn expression (Fig. 7b), indicating a decrease in inhibitory effect on autophagy in cells with low α -syn expression. It appears that exogenous dopamine not only leads to increase in basal expression of α -syn but also promotes its localization to cytoplasm and the inhibitory effect on autophagy by dopamine appears to be mediated via α -syn.

The cytotoxic effects of dopamine are countered by neurons by converting the excess dopamine to neuromelanin. As a common denominator in both neurons and melanoma, α -syn has been shown to decrease melanin in melanoma cells similar to the regulatory effect it has on dopamine (Pan et al. 2012). Since SK-MEL-28 contains both α -syn and melanin, the effect of dopamine on melanin content in presence of α -syn was assayed. Results showed that in the presence of exogenous dopamine, a 1.5-fold increase in melanin content was observed, despite the increase in α -syn levels induced by dopamine (Fig. 9). Therefore, it appears that these cells are able to

counter some of the toxic effects of dopamine such as oxidative stress through converting it to melanin, which is independent of the presence of α -syn. This increase in melanin, however, does not prevent the autophagy inhibition triggered by dopamine. Additionally, some studies report that inhibition of autophagy results in increased melanin content (Murase et al. 2013). We were also able to verify this with the combined treatment of Torin-1 and dopamine, which resulted in prolonged autophagy inhibition and in increased melanin content (Fig. 9).

Discussion

High cellular concentration of α -syn has been shown to trigger aggregation, impair degradation pathways, and cause oxidative damage leading to cytotoxicity (Hsu et al. 2000; Oliveras-Salva et al. 2013). On the contrary, a recent report demonstrates that at physiological levels of α -syn confers protection against oxidative damage and apoptosis (Musgrove et al. 2012). This suggests that protein levels in

Fig. 7 Effect of exogenous dopamine on α -syn. **a** Western blot and graph showing increase in α -syn when treated with 1 mM dopamine alone and in presence of 10 nM Torin-1 (T10D) at two time points of 8 and 24 h respectively ($n = 3$, $*p < 0.05$, compared to control). **b** Western blot and graph showing changes in autophagic marker p62 in cells treated with 1 mM dopamine in the presence and absence of α -syn for 8 h ($n = 3$, $*p < 0.05$)

itself are an important determinant of the switch between physiology and pathology. It is, therefore, important to contrast the behavior of α -syn between the overexpression and the endogenous models to understand the physiological roles of α -syn and to devise therapeutic strategies.

Dual localization of α -syn and effect of autophagy on α -syn expression and distribution

Our results with the melanoma cell model, SK-MEL-28, expressing endogenous α -syn reveal novel insights into the response of α -syn to changes in autophagy. Endogenous α -syn was recently reported to occur as a tetramer that resists aggregation although this view is debated (Bartels et al. 2011; Lashuel et al. 2013). Additionally, the presence of higher molecular weight species of α -syn in other cell types is also reported (Leng et al. 2001; Colasanti et al. 2014). In our study, not only did we observe the presence of a higher molecular weight species of α -syn, but we also demonstrated for the first time the differential localization of the

monomeric and the higher molecular weight α -syn. Although the localization of α -syn to the nucleus under physiological conditions remains controversial (Irizarry et al. 1996; Vivacqua et al. 2011), it is known to undergo nuclear localization only during conditions of cellular stress (Kontopoulos et al. 2006). However, in our study the monomeric α -syn appeared exclusively localized to the nucleus and was not found in the cytoplasm. A higher molecular weight oligomer was localized exclusively in the cytoplasm and was resistant to the denaturing conditions used in immunoblotting. Based on this observation, it is likely that the higher molecular weight α -syn seen here is non-toxic as it is present in the cell in physiological conditions. In vitro studies have shown that non-toxic α -syn oligomers exist when methionine residues are oxidized both at N terminal as well at the hydrophobic cluster at the C terminal (Zhou et al. 2010). While at present it is difficult to predict if the monomeric and oligomeric α -syn mediate different functions, there appears to exist a regular trafficking between the nucleus and the cytoplasm of the two

forms in response to different cellular conditions. Both autophagy inhibition and dopamine addition (possibly due to its inhibitory effect on autophagy) promoted cytoplasmic localization and appeared to favor the higher molecular weight species. This observation is consistent with other results that show that autophagy inhibition increases the secretion and release of α -syn (Lee et al. 2013b). Similarly, modifications such as phosphorylation have also been shown to promote α -syn localization to the cell surface and its subsequent release (Lee et al. 2013a).

Another significant observation from this study is the response of monomeric α -syn to autophagy induction. While overexpressed α -syn has been shown to impair macroautophagy (Winslow et al. 2010), results from studies on T-lymphocytes suggest that α -syn has a regulatory effect on autophagy (Colasanti et al. 2014). We

were able to demonstrate a similar effect in melanoma cells. Monomeric α -syn did not completely impair autophagy, and the cells continued to respond to autophagy induction although in absence of α -syn they showed a heightened response to autophagy induction. This strongly suggests that physiological α -syn has a regulatory role in autophagy without resulting in impairment of autophagy. Because this phenomenon has now been independently verified in SK-MEL-28 as well, it is plausible that α -syn may have a similar physiological function in neuronal cells.

Effect of exogenous dopamine on α -syn expression and distribution

Among the various cellular factors that influence a constitutive pathway such as autophagy, we found

Fig. 8 Immunostaining of α -syn. **a** SK-MEL-28 cells stained for α -syn after treatment with Torin-1 at concentrations of 2 and 10 nM for 12 h showing an increase in nuclear staining when compared with control. **b** SK-MEL-28 cells stained for α -syn after treatment with 10 and 20 μ M Chloroquine for 12 h shows an increase in cytoplasmic localization of α -syn when compared with control. Inset shows enlarged image of a single cell. **c** SK-MEL-28 cells stained for α -syn after treatment with 1 mM dopamine shows an increase in cytoplasmic localization of α -syn. Inset for each image shows enlarged image of a single cell. (Scale bar for all images = 50 μ m)

Fig. 9 Changes in melanin content. Increase in melanin content in cells treated with 1 mM dopamine when compared to control while a combination of dopamine and 10 nM Torin-1 did not lead to a similar increase at 8 h. Drop in melanin content observed in cells treated with

1 mM dopamine after 16 h while combined treatment of dopamine and Torin-1 leads to an increase in melanin content by 16 h ($n = 3$, $p < 0.05$) where T10D represents combined treatment of 10 nM Torin-1 and 1 mM dopamine

dopamine to exert a negative effect on autophagy even when supplied exogenously and in the face of high resistance shown by SK-MEL-28 cells. Although these cells appeared to combat the effect of dopamine-mediated oxidative stress, they could not prevent the effect it had on autophagy. The conversion of dopamine to neuromelanin is a mechanism by which neurons combat dopamine-induced toxicity in cells. However despite these measures, dopamine metabolism is known to result in oxidative damage, which when accumulated over time contributes to pathogenesis (Segura-Aguilar et al. 2014). Interestingly, despite the lack of oxidative stress in the melanoma cells, dopamine in the presence of α -syn had an inhibitory effect on autophagy, suggesting additional pathological effects of dopamine. This inhibition of autophagy over prolonged periods of time may prove costly to cells, particularly in neurons that rely so heavily on autophagy to maintain cellular homeostasis (Glick et al. 2010). This could be a possible mechanism for α -syn aggregation in neuronal cells. Therefore, the induction of autophagy through chemical means (such as with rapamycin) has been widely studied, with the belief that it can counter situations of autophagy inhibition by reversing it. Given the regulatory effect α -syn has on autophagy, as demonstrated in the present study, enhancing autophagy appears to be counter-productive to the cell. The presence of α -syn prevented the induction of autophagy. Additionally, exposure of cells to dopamine combined with autophagy induction resulted in further increases in basal α -syn levels, considerably enhancing the protein burden on the cell. This resulted in the sustained inhibition of autophagy and was accompanied with increase in melanin content. This has consequences for PD pathogenesis, where prolonged autophagy inhibition can potentially result in excess

neuromelanin that is found to be cytotoxic (Segura-Aguilar et al. 2014). It, therefore, appears that autophagy induction can compromise cell survival under certain conditions, and in light of this new evidence, the efficacy of autophagy enhancement, as a therapeutic tool, needs to be carefully examined.

Relevance of melanoma model

A strong correlation between PD and melanoma has been demonstrated by epidemiological studies (Pan et al. 2011), and in vitro studies have shown the transfer of α -syn from melanoma to neuroblastoma cells in co-culture (Hansen et al. 2011). A recent report studying α -syn behavior in SK-MEL-28 proposes that the transfer of α -syn from melanoma to neuronal cells may affect the pathogenesis of PD (Lee et al. 2013a). Interestingly, the transplantation of tyrosinase-positive melanocytes in rat models of PD has been shown to have beneficial effects and further argues for the need to explore α -syn behavior in melanoma (Asanuma et al. 2013). The presence of higher molecular weight species of α -syn in SK-MEL-28 and the differential localization of the two forms of α -syn makes SK-MEL-28 a good model to evaluate the formation and existence of non-toxic oligomers and to investigate if these higher order structures have any physiological significance under normal cellular conditions. Role of chaperone-mediated autophagy and proteasomal pathway in clearance of monomeric and higher molecular weight α -syn seen in this cell line also needs to be elucidated.

Conclusion

It remains to be verified if these observations hold true in neuronal systems, yet studies on alternate cell models allow us to arrive at a more comprehensive understanding of the behavior of α -syn and its effect

on PD pathogenesis. The inverse relation exhibited between α -syn and autophagy can negatively affect autophagy when used as a treatment strategy. These observations gain significance while developing therapeutic interventions, given the wide expression of α -syn in non-neuronal cells.

α -syn, α -Synuclein; ALP, Autophagy-Lysosomal Pathway; PD, Parkinson's disease; UPS, Ubiquitin-Proteasome System.

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Author contribution SN, AK, and AT were involved in the concept and design of experiments, data interpretation, and manuscript preparation. SN and BV carried out the experiments.

Compliance with ethical standards

Conflict of interest No conflicts of interest to declare.

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