

## ORIGINAL ARTICLE

# Latrepirdine stimulates autophagy and reduces accumulation of $\alpha$ -synuclein in cells and in mouse brain

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Latrepirdine (Dimebon; dimebolin) is a neuroactive compound that was associated with enhanced cognition, neuroprotection and neurogenesis in laboratory animals, and has entered phase II clinical trials for both Alzheimer's disease and Huntington's disease (HD). Based on recent indications that latrepirdine protects cells against cytotoxicity associated with expression of aggregatable neurodegeneration-related proteins, including A $\beta$ 42 and  $\gamma$ -synuclein, we sought to determine whether latrepirdine offers protection to *Saccharomyces cerevisiae*. We utilized separate and parallel expression in yeast of several neurodegeneration-related proteins, including  $\alpha$ -synuclein ( $\alpha$ -syn), the amyotrophic lateral sclerosis-associated genes *TDP43* and *FUS*, and the HD-associated protein huntingtin with a 103 copy-polyglutamine expansion (*HTT* gene; htt-103Q). Latrepirdine effects on  $\alpha$ -syn clearance and toxicity were also measured following treatment of SH-SY5Y cells or chronic treatment of wild-type mice. Latrepirdine only protected yeast against the cytotoxicity associated with  $\alpha$ -syn, and this appeared to occur via induction of autophagy. We further report that latrepirdine stimulated the degradation of  $\alpha$ -syn in differentiated SH-SY5Y neurons, and in mouse brain following chronic administration, in parallel with elevation of the levels of markers of autophagic activity. Ongoing experiments will determine the utility of latrepirdine to abrogate  $\alpha$ -syn accumulation in transgenic mouse models of  $\alpha$ -syn neuropathology. We propose that latrepirdine may represent a novel scaffold for discovery of robust pro-autophagic/anti-neurodegeneration compounds, which might yield clinical benefit for synucleinopathies including Parkinson's disease, Lewy body dementia, rapid eye movement (REM) sleep disorder and/or multiple system atrophy, following optimization of its pro-autophagic and pro-neurogenic activities.

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## INTRODUCTION

Latrepirdine (Dimebon; dimebolin) is a neuroactive compound with antagonist activity at histaminergic,  $\alpha$ -adrenergic and serotonergic receptors that was associated with enhanced cognition,<sup>1–4</sup> neuroprotection<sup>5,6</sup> and neurogenesis<sup>7</sup> in laboratory animals. Based on its effects on cognition in rodents and its highly favorable safety profile, the compound entered clinical trials for both Alzheimer's disease (AD)<sup>8</sup> and Huntington's disease (HD).<sup>9</sup> Related reports indicate that latrepirdine protects against the cytotoxicity associated with A $\beta$ 42 (ref. 10) or  $\gamma$ -synuclein<sup>11</sup> by stimulating catabolism of these aggregation-prone, neurodegeneration-related proteins. Here, we sought to determine whether latrepirdine offered protection against the cytotoxicity associated with the accumulation of several neurodegeneration-related proteins including  $\alpha$ -synuclein ( $\alpha$ -syn), the amyotrophic lateral

sclerosis-associated genes *TDP43* and *FUS*, and the HD-associated protein huntingtin with a 103 copy-polyglutamine (polyQ) expansion (*HTT* gene; htt-103Q). We report that latrepirdine improved cell viability in *Saccharomyces cerevisiae* (*S. cerevisiae*) expressing  $\alpha$ -syn, but offered no protection to yeast strains expressing *TDP43*, *FUS* or htt-103Q.

Parkinson's disease (PD) and AD are the two most common forms of neurodegenerative diseases. Both PD and AD patients present with progressive cognitive decline resulting from a pathogenic accumulation of insoluble protein aggregates that precedes region-specific synaptic and neuronal loss.<sup>12</sup> Formation of intracellular Lewy bodies, which consist primarily of  $\alpha$ -syn and ubiquitin, is associated with the pathogenesis of PD, Lewy body dementia, rapid eye movement (REM) sleep disorder and multiple system atrophy.<sup>12</sup> Interestingly, 30–50% of AD patients also harbor atypical  $\alpha$ -syn

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pathology (in addition to canonical AD neuropathology) in the cerebral cortex.<sup>13,14</sup> Indeed,  $\alpha$ -syn was originally identified as the non-A $\beta$  component of AD-related senile plaques, and was later determined to represent a primary component of Lewy bodies.<sup>12,15</sup>

Recent studies suggest that  $\alpha$ -syn exists as a stable helically folded tetramer that must undergo destabilization before misfolding and formation of the fibrillar aggregates that are observed in synucleinopathies.<sup>16</sup> We hypothesized that a compound that protects against the cytotoxicity associated with  $\alpha$ -syn might provide clinical benefit to patients harboring synucleinopathies, including PD, Lewy body dementia, REM sleep disorder, multiple system atrophy and in some instances of AD.

To date, the most promising regulators of  $\alpha$ -syn degradation and protection against  $\alpha$ -syn-induced cytotoxicity are small molecule enhancers of rapamycin (SMER; most notably SMER-28). Recently, SMER-28 was shown to induce autophagy, improve cell viability and promote clearance in cellular models of neurodegenerative disease-related proteins including amyloid precursor protein metabolites (among them A $\beta$ <sup>17</sup>), htt<sup>18</sup>, and  $\alpha$ -syn.<sup>18</sup> In related studies, we reported that latrepirdine protected against cytotoxicity associated with A $\beta$ 42 accumulation in *S. cerevisiae*,<sup>10</sup> in mammalian cells and in the brains of transgenic CRND8 mice<sup>19</sup> via induction of mammalian target of rapamycin- and Atg5-dependent autophagy. Here, we provide evidence to indicate that latrepirdine: (1) protects against  $\alpha$ -syn-related cytotoxicity; (2) enhances autophagy and degradation of  $\alpha$ -syn in three different model systems; and (3) when chronically administered, reduces  $\alpha$ -syn levels in mouse brain in parallel with the induction of autophagy.

## MATERIALS AND METHODS

### Preparation and handling of latrepirdine

The synthesis and characterization of latrepirdine was described previously.<sup>20</sup> Briefly, latrepirdine was purchased from SinoChemexper (Shanghai, China) and purity of the compound was determined to be >99% or provided directly by Medivation, San Francisco, CA, USA. For use *in vitro*, latrepirdine was dissolved directly into culture media to the desired concentration, as described in Steele *et al.*<sup>20</sup> For administration *in vivo*, latrepirdine was dissolved into 0.9% saline (vehicle) at a final concentration of 3.5 mg ml<sup>-1</sup> (made fresh every 2 days).

### Yeast growth curve and spotting assay

Yeast cells were grown at 30 °C unless specifically mentioned. Growth of yeast strains was monitored using Bioscreen ([www.bioscreen.fi](http://www.bioscreen.fi)). Yeast strains were pre-grown in 2% raffinose, diluted to an OD<sub>600</sub> of 0.05 and induced with 2% galactose. Optical density (OD) measurements were taken every 1 h. Raw data were averaged among independent experiments and OD<sub>600</sub> plotted overtime as a growth curve. For spotting assays, yeast cells were grown overnight to mid-log phase in raffinose medium. Cultures were then normalized to OD<sub>600</sub>=5.0, and 10 $\times$  serially diluted and spotted onto the respective dropout plates containing 2% glucose or galactose. Pictures of plates were taken after 2–3 days of growth.

### Assay of nonspecific autophagy in yeast

Yeast strain YTS158 (generous gift from Daniel J Klionsky, University of Michigan) was grown in the absence or presence of different concentrations of latrepirdine (as indicated) to mid-log phase. Cells were then harvested and alkaline phosphatase activity was measured using a spectrophotometric assay as described.<sup>21</sup>

### SH-SY5Y cell culture experiments

SH-SY5Y cells stably transfected with a doxycycline (dox)-inducible ('tet-off') wild-type  $\alpha$ -syn gene, and control SH-SY5Y cells overexpressing the  $\beta$ -galactosidase gene, (a gift from L Stefanis, Division of Basic Neurosciences, Biomedical Research Foundation of the Academy of Athens,

Athens, Greece)<sup>22</sup> were cultured and treated with or without drug. See Supplemental Methods section for detailed methods.

### Experimental animals and drug treatment

All mice in this study were maintained on a hybrid C3H/He-C57BL/6 background (referred to here as 'wild-type' mice). Male 2-month-old littermates received 21 consecutive once-daily intraperitoneal injections of either 3.5 mg kg<sup>-1</sup> latrepirdine ( $n=4$ ) or 0.9% saline (vehicle;  $n=4$ ). Following treatment, animals were killed and transcardially perfused with ice-cold phosphate-buffered saline (pH 7.4). Brains were dissected and snap-frozen for biochemical analysis. Animals were individually housed and maintained on a 12:12 light:dark cycle (lights on at 0700 hours) with *ad libitum* access to food and water throughout the course of the entire experiment. All experimental protocols described herein were conducted within NIH guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine. See Supplemental Methods for detailed methods.

### Statistical analysis

Integrated density of immunoreactive western blot bands was measured using MultiGauge Software (Fujifilm Life Sciences, Stamford, CT, USA) and normalized to % control (vehicle or nTg littermate, where indicated). In all instances, Shapiro–Wilk test for normality of distribution and Levene's test for homogeneity of variance were utilized for inclusion in parametric tests ( $P>0.05$  for Shapiro–Wilk and Levene's tests). Independent samples *t*-tests (parametric design) or Mann–Whitney *U*-tests (non-parametric design) were utilized to determine significant mean differences between two groups. Significance for *t*-tests and analysis of variances are reported with a  $P\leq 0.05$  using two-tailed tests with an  $\alpha$ -level of 0.05. All statistical analyses were performed using SPSS v18.0 (IBM North America, New York, NY, USA) and/or GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

## RESULTS

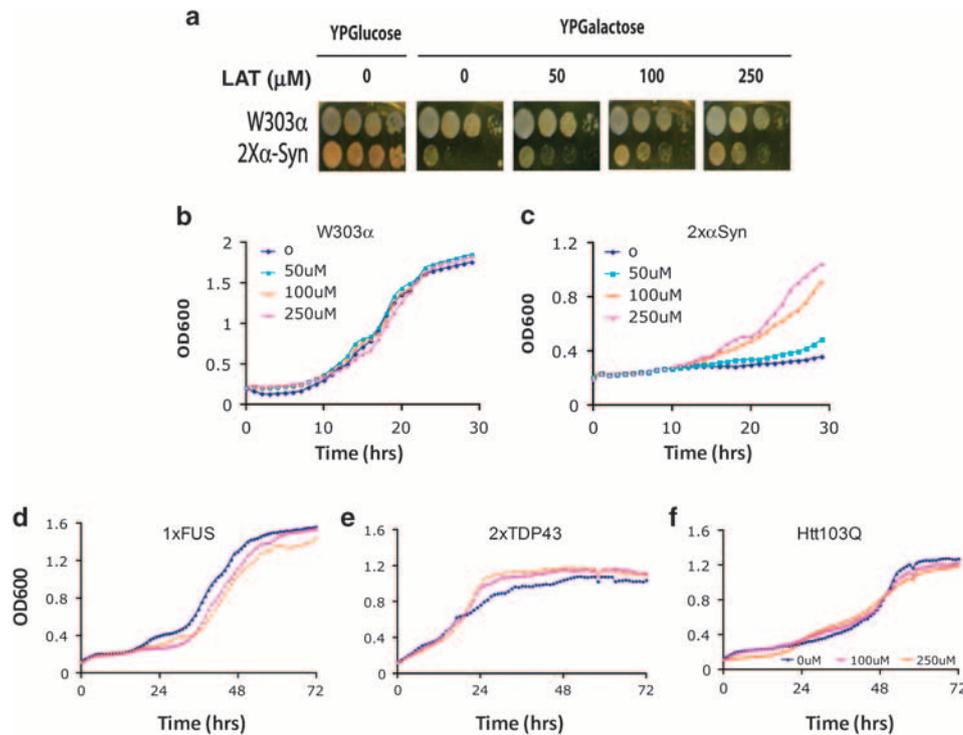
### Latrepidine protected *S. cerevisiae* against $\alpha$ -syn toxicity

We used an *S. cerevisiae* model system to investigate whether latrepirdine could protect from  $\alpha$ -syn or any of a panel of proteotoxic species associated with neurodegenerative diseases (Table 1). Integration of a single copy of  $\alpha$ -syn in *S. cerevisiae* (1X $\alpha$ Syn) had no appreciable effect on cellular growth.<sup>23</sup> However, an increase in  $\alpha$ -syn gene dosage from one to two copies (2X $\alpha$ Syn) resulted in growth arrest and cell death.<sup>23</sup> To test the possibility that yeast could be protected from  $\alpha$ -syn toxicity by latrepirdine, we monitored the growth of the 2X $\alpha$ Syn strain and the isogenic wild-type (W303 $\alpha$ ) strain in the presence of different concentrations of latrepirdine (Figures 1a–c). Latrepirdine treatment was associated with sustained viability of the 2X $\alpha$ Syn strain, with no effect on growth of the W303 $\alpha$  strain. We conclude that latrepirdine treatment was associated with sustained cell viability in the face of  $\alpha$ -syn overexpression, which may be related to an effect of the drug on protein degradation.

To test whether the protection from proteotoxicity by latrepirdine was specific to  $\alpha$ -syn, we also studied the effect of latrepirdine on other *S. cerevisiae* models of proteotoxicity in neurodegenerative diseases, including the amyotrophic lateral sclerosis-associated genes *TDP43* (2XTDP43) and *FUS* (1XFUS), and the HD-associated htt-103Q. Despite the well-established properties of each of these proteins to form aggregates that are toxic to yeast, latrepirdine offered specific protection from only the proteotoxicity of  $\alpha$ -syn (Figures 1a–c); that is, the drug was unable to afford protection from the proteotoxicity associated with FUS, TDP43 or Htt-103Q (Figures 1d–f). One factor underlying the heterogeneity of proteotoxicity may be attributable to the different intracellular compartments in which the aggregates accumulate. Heterogeneity of proteotoxicity also occurs within the same polypeptide as exemplified by the prion protein strain phenomenon, in which some aggregates of prion protein are

**Table 1.** Yeast strains used in this study

Strains	Genotype	Reference
W303 $\alpha$	MATAade2-1can1-100his3-11,15leu2-3112,trp1-1, ura3-1	Cooper <i>et al.</i> <sup>31</sup>
2X $\alpha$ Syn	Integration of $\alpha$ -synuclein into HIS3 and TRP1 loci of W303 $\alpha$ (IntTox)	Cooper <i>et al.</i> <sup>31</sup>
1XFUS	Integration of FUS/TLS into TRP1 locus of W303 $\alpha$	Ju <i>et al.</i> <sup>32</sup>
2XTDP43	Integration of TDP43 into HIS3 and TRP1 loci of W303 $\alpha$	Johnson <i>et al.</i> <sup>33</sup>
Htt103Q	Integration of N-terminal huntingtin fragment with stretch of 103 glutamine into HIS3 locus of W303 $\alpha$	Willingham <i>et al.</i> <sup>34</sup>
YTS158	BY4742 with integration of pho8 $\Delta$ ::pho8 $\Delta$ 60(URA3) pho13 $\Delta$ ::Kan	He <i>et al.</i> <sup>35</sup>
Atg8 $\Delta$ 2X $\alpha$ Syn	2X $\alpha$ Syn with integration of atg8 $\Delta$ ::LEU2	This study



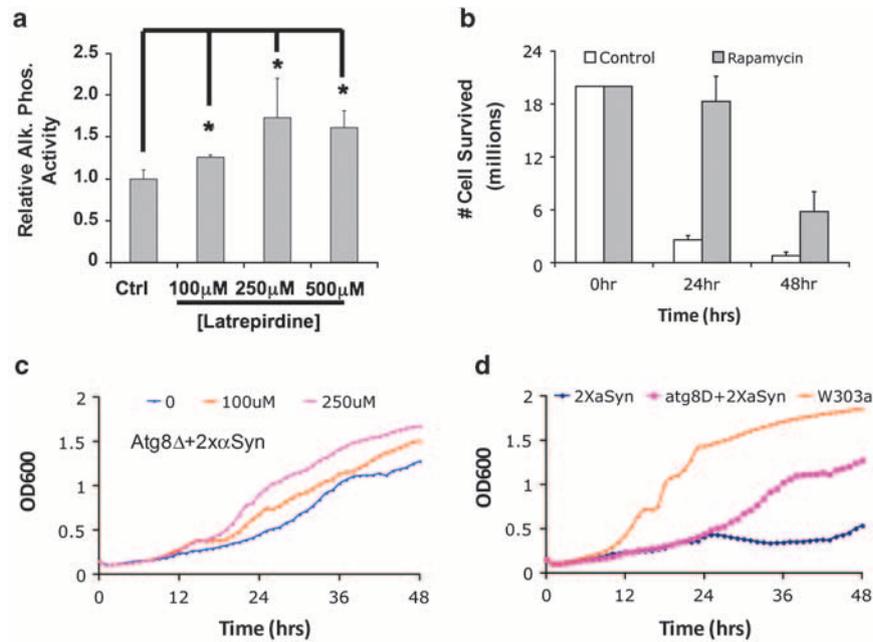
**Figure 1.** Latrepirdine protects *S. cerevisiae* from cytotoxicity of  $\alpha$ -synuclein ( $\alpha$ -syn), but not TDP-43, FUS or Htt-103Q. (a) 2X $\alpha$ Syn strain and its isogenic wild-type (W303 $\alpha$ ) strains were grown to mid-log phase in raffinose medium, diluted and then spotted onto YPGlucose plate ( $\alpha$ -syn is 'off') and YPGalactose plates ( $\alpha$ -syn 'on') in the absence or presence of latrepirdine (pictures were taken 2 days after growth at 30 °C). (b) W303 $\alpha$  or (c) 2X $\alpha$ Syn strains were grown in YPGalactose medium ( $\alpha$ -syn is 'on') in the absence or presence of latrepirdine (concentration as indicated). (d) 1XFUS, (e) 2XTDP43 and (f) 1XHtt103Q strains were grown in YPGalactose medium (expression is 'on') in the absence or presence of different concentrations of latrepirdine (as indicated). All figures are representative of three or more independent experiments.

benign and well-tolerated by cells while other aggregates are toxic and pathogenic for transmissible spongiform encephalopathies.<sup>24</sup> Either or both of these phenomena might explain, at least in part, the differential rescue of yeast from some, but not all toxic aggregates. In related studies, we reported that latrepirdine protects yeast against proteotoxicity associated with A $\beta$ 42 accumulation via induction of autophagy,<sup>10</sup> as well as in mammalian cell lines and in mouse brain.<sup>19</sup> The specificity of latrepirdine for  $\alpha$ -syn shown here, taken together with these two reports, suggests that fibrillar assemblies formed by  $\alpha$ -syn or A $\beta$ 42 aggregates may be preferentially targeted for degradation by the autophagic pathway following latrepirdine stimulation.

Genetic and pharmacological evidence indicate that latrepirdine-mediated protection of yeast from  $\alpha$ -syn toxicity involved autophagy

We sought to determine whether latrepirdine protection of yeast from  $\alpha$ -syn toxicity was attributable to activation of autophagy—a

conserved pathway regulating the degradation of long-lived proteins, cellular organelles and protein aggregates. Addition of rapamycin to yeast cultures strongly induced autophagy, even in a nutrient-rich medium.<sup>25</sup> Using a yeast reporter of non-specific autophagy (in which maturation of the autophagosome activated an alkaline phosphatase; YTS158 strain),<sup>21</sup> we observed a significant and dose-dependent increase in alkaline phosphatase activity following treatment with latrepirdine, suggesting that latrepirdine activates autophagy (Figure 2a). As we observed latrepirdine-induced activation of mammalian target of rapamycin-dependent autophagy in mammalian cell lines and mouse brain,<sup>19</sup> we sought to determine whether activation of target of rapamycin-dependent autophagy could also protect the 2X $\alpha$ Syn strain from  $\alpha$ -syn toxicity. Treatment of yeast with rapamycin (10  $\mu$ M) was also associated with a significant increase in the viability of 2X $\alpha$ Syn cells, further confirming that stimulation of autophagic activity was sufficient to prevent the proteotoxicity associated with  $\alpha$ -syn aggregation in the 2X $\alpha$ Syn model (Figure 2b).



**Figure 2.** Latrepirdine or rapamycin protect against  $\alpha$ -synuclein ( $\alpha$ -syn)-related cytotoxicity via induction of autophagy. (a) In the YTS158 strain, maturation of the autophagosome results in activation of a functional alkaline phosphatase. Alkaline phosphatase activity was measured from YTS158 stain following growth in synthetic medium to log phase in the absence or presence of the indicated concentrations of latrepirdine (as indicated). (b)  $2x\alpha$ Syn strains were grown in YPGalactose medium ( $\alpha$ -syn is 'on') in the absence or presence of rapamycin (concentration as indicated). (c)  $Atg8\Delta + 2X\alpha$ Syn cultures were grown in YPGalactose medium ( $\alpha$ -syn is 'on') in the absence or presence of different concentrations of latrepirdine (as indicated) and growth was monitored by measuring OD600. (d)  $2X\alpha$ Syn, its isogenic wild type (W303 $\alpha$ ), and  $2X\alpha$ Syn with deletion of the *ATG8* gene ( $Atg8\Delta + 2X\alpha$ Syn) were grown in YPGalactose ( $\alpha$ -syn is 'on') medium at 30 °C with shaking. Growth of cells was monitored by OD600 at an interval of 1 h using a Bioscreen machine. All figures are representative of three or more independent experiments, performed in duplicate or triplicate. Graphs are mean  $\pm$  s.e.m.; \* $P < 0.05$ .

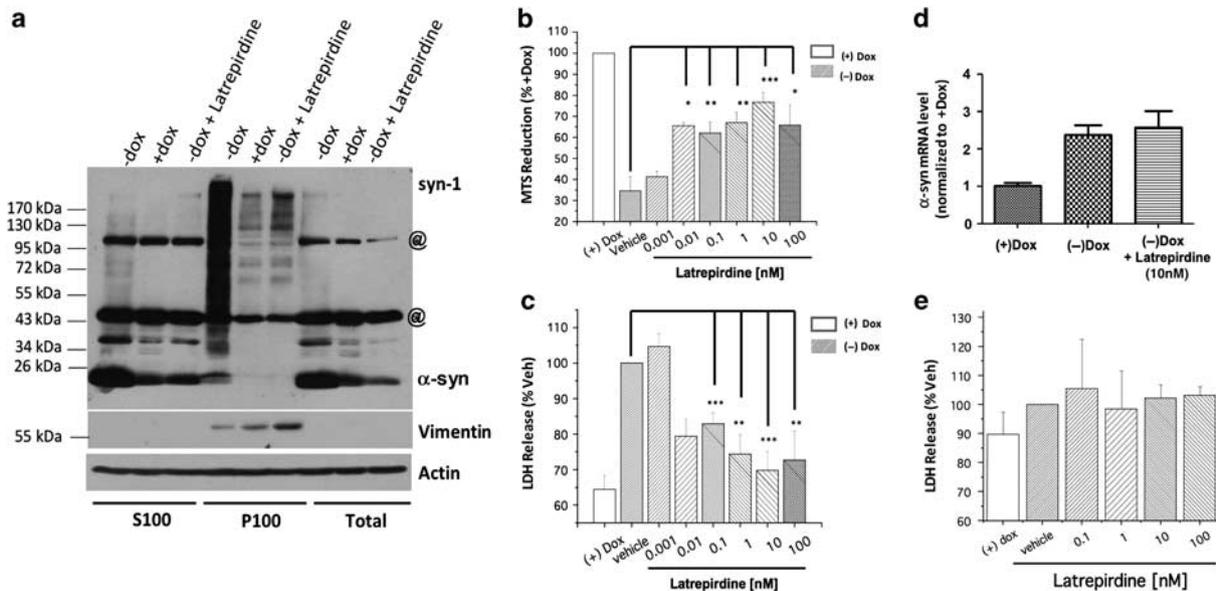
*ATG8* (yeast homolog of LC3) encodes an ubiquitin-like protein required for autophagosome formation. In order to test the hypothesis that latrepirdine-mediated protection from  $\alpha$ -syn toxicity involved autophagy, we crossed the  $2X\alpha$ Syn strain with an *atg8 $\Delta$ ::LEU2* strain ( $Atg8\Delta 2X\alpha$ Syn; Table 1).  $Atg8\Delta 2X\alpha$ Syn cultures were grown in the absence or presence of 100  $\mu$ M or 250  $\mu$ M latrepirdine, and growth was monitored by OD600 (Figure 2c). Although 100  $\mu$ M latrepirdine was associated with protection from  $\alpha$ -syn toxicity in the  $2X\alpha$ Syn strain (Figures 1a–c), this concentration was insufficient to protect the  $Atg8\Delta 2X\alpha$ Syn strain (Figure 2c). However, higher concentrations of latrepirdine (250  $\mu$ M) appeared to offer partial protection from  $\alpha$ -syn toxicity in  $Atg8\Delta$  (Figure 2c). This observation suggests latrepirdine may protect cells against  $\alpha$ -syn-related toxicity through at least one pathway that involves the regulation of *Atg8*-dependent autophagy. In contrast to prior observations,<sup>18</sup> ablation of *Atg8*-dependent autophagy resulted in increased cell viability of  $Atg8\Delta 2X\alpha$ Syn after 24 h of growth, by comparison with  $2X\alpha$ Syn cells, although both exhibited less viability when compared with the isogenic wild-type strain at all-time points (Figure 2d). We propose that a compensatory mechanism that is selective for survival of the  $Atg8\Delta 2X\alpha$ Syn strain may be induced after 24 h of growth, allowing for modest improvement in cell viability. In future studies, we plan to investigate these pathways in more detail in order to understand the mechanisms underlying improved survival after 24 h in the autophagy-deficient  $Atg8\Delta 2X\alpha$ Syn strain.

Latrepidine promoted cell viability and protected against  $\alpha$ -syn toxicity in differentiated SH-SY5Y neurons

We tested latrepirdine altered  $\alpha$ -syn aggregation using a stably expressing, inducible tet-off system to overexpress wild-type human  $\alpha$ -syn in differentiated SH-SY5Y neurons. In this system,

$\alpha$ -syn expression is off in the presence of dox and switched on in cells deprived of dox. We analyzed the levels of monomeric and oligomeric  $\alpha$ -syn in total lysates, Triton X-100 soluble and insoluble protein fractions. Total levels of  $\alpha$ -syn protein were higher than in control cells maintained in the  $\alpha$ -syn off (+ dox) state. We observed that treatment with 10 nM latrepirdine by 14 days decreased the levels of all forms of  $\alpha$ -syn, including aggregates and soluble monomer in the Triton X-100 soluble, total and insoluble fractions (Figure 3a). Similar results in western blots were obtained using the LB509 anti- $\alpha$ -syn antibody (data not shown). However, no significant differences were found in the levels of  $\alpha$ -syn mRNA measured by quantitative reverse transcriptase-quantitative PCR at 14 days of culture (Figure 3d), suggesting that latrepirdine regulated  $\alpha$ -syn levels via a mechanism that was probably post-translational. This is consistent with a role for protein degradation (for example, autophagy) in  $\alpha$ -syn catabolism.

We observed that differentiated SH-SY5Y neurons grown in the absence of dox for 14 days (overexpressing  $\alpha$ -syn), exhibited increased necrotic cell death (Figures 3b and c). This conclusion was supported by the observation of a  $\sim$ 36% rise in levels of lactate dehydrogenase (LDH) released into the culture medium in cells overexpressing  $\alpha$ -syn as compared with cells maintained in medium containing dox (Figure 3c). In addition, we determined the viability of the remaining non-necrotic cell by measuring the capacity of these cells to metabolize the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) into a soluble formazan. Reduction in the capacity of MTS metabolism is an indicator of cell injury. We found that latrepirdine treatment decreased cell death of  $\alpha$ -syn overexpressing cells as measured by LDH release (Figure 3c) and increased their viability as indicated by their capacity to reduce MTS (Figure 3b). This cytoprotective effect of



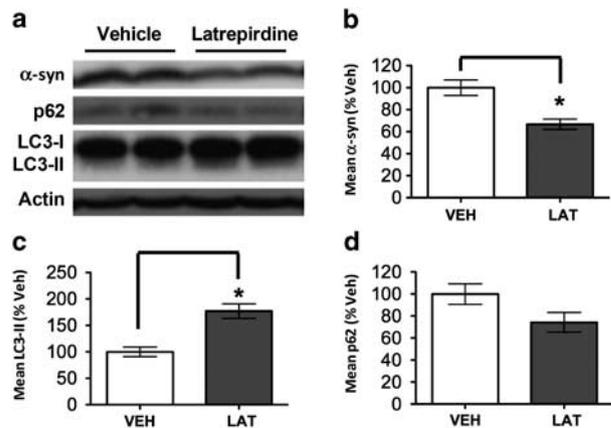
**Figure 3.** Latrepirdine stimulates the catabolism of  $\alpha$ -synuclein ( $\alpha$ -syn) and protects against  $\alpha$ -syn-related cytotoxicity in differentiated SH-SY5Y neurons. SH-SY5Y cells stably expressing inducible ('tet-off') human  $\alpha$ -syn were differentiated (without doxycycline (dox)) using retinoic acid and treated in the absence (vehicle; without dox) or presence of latrepirdine (without dox) for 14 days. **(a)** Cells treated for 14 days in the presence of 10 nM latrepirdine were lysed and Triton X-100 soluble (S100, left) and insoluble (P100, middle) pellets were obtained by ultracentrifugation ('@' are nonspecific bands). Treatment with latrepirdine decreased the levels of all forms of  $\alpha$ -syn, including aggregates and soluble monomer in the Triton X-100 soluble (monomer = 52.7% reduction; s.e.m. = 10.38;  $P < 0.01$ ), total (monomer = 68.0% reduction; s.e.m. = 15.64;  $P < 0.01$ ), and insoluble fractions (aggregated = 74.8% decrease; s.e.m. = 9.397;  $P < 0.001$ ). **(b)** Quantification of lactate dehydrogenase (LDH) released to the culture medium or **(c)** measurements of tetrazolium MTS reduction in SH-SY5Y cells was used to determine cell viability in the absence of presence of a range of concentrations of latrepirdine (as indicated). **(d)**  $\alpha$ -Syn mRNA levels were analyzed using quantitative reverse transcriptase-PCR, normalized to  $\alpha$ -tubulin mRNA levels. **(e)** Inducible SH-SY5Y cells overexpressing  $\beta$ -galactosidase ( $\beta$ -gal) were cultured in the absence of dox in 96-well plates and treated after 24 h with retinoic acid (RA) and different concentrations of latrepirdine (as indicated), and LDH release was quantified from the culture medium. All figures are representative of three or more independent experiments. Graphs are mean  $\pm$  s.e.m.; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

latrepirdine treatment was observed with concentrations of latrepirdine as low as 0.1 nM. As a control, we also measured cell death and viability in SH-SY5Y differentiated cells overexpressing  $\beta$ -galactosidase in the absence (with or without dox) or presence of latrepirdine. These cells did not display significant changes in cell death or viability (Figure 3e).

Chronic latrepirdine treatment was associated with enhanced autophagy and reduction of  $\alpha$ -syn monomer in the brains of wild-type mice

In a related paper,<sup>19</sup> we provided evidence that chronic latrepirdine (3.5 mg kg<sup>-1</sup> per day) administration enhanced autophagy, reduced accumulation of A $\beta$ 42 and improved behavioral impairment in an AD mouse model. We next investigated whether chronic administration of latrepirdine to wild-type mice for 3 weeks was sufficient to activate autophagy and/or to alter  $\alpha$ -syn levels in their brains. Male wild-type mice received 21 once-daily intraperitoneal injections of either latrepirdine (3.5 mg kg<sup>-1</sup>;  $n = 4$ ) or vehicle (0.9% saline;  $n = 4$ ). Following treatment, mice were killed and whole-brain lysates were analyzed for autophagy markers, including LC3 and the polyubiquitin binding protein p62/SQSTM1 (p62), and  $\alpha$ -syn levels by western blot (Figure 4). LC3 is converted from its cytosolic precursor (LC3-I) to its membrane-associated form (LC3-II) by the addition of phosphatidylethanolamine, which allows for the recruitment of LC3-II to the outer membrane of the autophagosome.<sup>26</sup> When autophagy is induced, autophagosomes accumulate and mature until terminal degradation on fusion with the lysosome.<sup>26</sup>

Following 21 days of latrepirdine treatment, we noted a significant decrease in  $\alpha$ -syn monomer in the brains of WT mice,



**Figure 4.** Latrepirdine stimulates  $\alpha$ -synuclein ( $\alpha$ -syn) catabolism in parallel to markers of enhanced autophagy. Two-month-old wild-type male mice received 21 consecutive once daily intraperitoneal injections of 3.5 mg kg<sup>-1</sup> latrepirdine ( $n = 4$ ; LAT) or equivalent volume of vehicle (0.9% saline;  $n = 4$ ; VEH). **(a)** Western blot analysis of wild-type mouse brains for protein levels of soluble  $\alpha$ -syn, p62 and LC3. **(b–d)** Quantification of western blot band integrated density. Graphs are representative littermates; mean  $\pm$  s.e.m.; \* $P < 0.05$ .

and this was associated with significantly elevated LC3-II accumulation but without accumulation of p62. These results support the hypothesis that latrepirdine stimulates  $\alpha$ -syn degradation *in vivo* in the WT mouse brain, which is likely via induction of autophagy,

6 an observation that dovetails with our recently reports.<sup>10,19</sup> Furthermore, the current results suggest that the turnover of  $\alpha$ -syn monomer may be determined, at least in part, by autophagic stasis, raising the possibility that enhanced autophagic activity might mitigate the toxicity associated with cellular accumulation of  $\alpha$ -syn. Ongoing experiments with latrepirdine in cellular and animal models expressing human  $\alpha$ -syn will help to elucidate the specific mechanisms of latrepirdine action on regulation of the autophagic pathway with regard to clearance of pathological  $\alpha$ -syn.

## DISCUSSION

Protein aggregates are resistant to degradation and therefore inherently longer-lived, allowing for sustained toxicity associated with the buildup of these cytotoxic assemblies.<sup>27</sup> Based on this principle, a wealth of studies have recently investigated the potential of protein catabolism pathway as therapeutic targets for several neurodegenerative diseases, including AD, HD and PD. Evidence indicates that  $\alpha$ -syn is a substrate of autophagy—a conserved catabolic pathway for the degradation of long-lived proteins, cellular organelles and protein aggregates. Studies of  $\alpha$ -syn clearance indicate that  $\alpha$ -syn is primarily degraded by the ubiquitin–proteasome system under normal conditions *in vivo*; however, autophagy may be the primary clearance pathway in cases of  $\alpha$ -syn overexpression or for the degradation of oligomeric  $\alpha$ -syn, which may be too large to fit into the narrow pore of the ubiquitin–proteasome system.<sup>28</sup> Cellular studies also indicate that  $\alpha$ -syn catabolism can be regulated by SMERs, which nonspecifically increase Atg5-dependent autophagy.<sup>18</sup> Another report suggests that conditional deletion of *ATG7* results in the accumulation of  $\alpha$ -syn aggregates in dopaminergic neurons of mice.<sup>29</sup>

Herein, we report that latrepirdine, a neuroactive compound with a compelling clinical safety profile, protects *S. cerevisiae* and differentiated SH-SY5Y neurons against  $\alpha$ -syn-induced (or A $\beta$ 42-induced)<sup>10</sup> cytotoxicity. Interestingly, *S. cerevisiae* expressing TDP43, FUS or htt-103Q were not protected by latrepirdine in these assays and these findings (specifically regarding htt-103Q) are partially validated by the recent failure of latrepirdine in a phase II trial for HD.<sup>9</sup> Taken together, we suggest that the latrepirdine-related protection against  $\alpha$ -syn cytotoxicity is likely due to promotion of a conserved catabolic pathway (for example, autophagy). We provide evidence here (Figures 2 and 4), and elsewhere,<sup>10,19</sup> that latrepirdine stimulates autophagy, resulting in the degradation of  $\alpha$ -syn and increased cell viability in yeast, in differentiated SH-SY5Y neurons, and *in vivo* in mouse brains. Based on these findings, we propose that latrepirdine may represent a potentially viable lead compound that might yield clinical benefit for synucleinopathies following optimization of its pro-autophagic and/or pro-neurogenic activity.

Recently, latrepirdine failed in a US-based phase II replication trial<sup>30</sup> of a prior successful Russian phase II trial of mild-to-moderate AD.<sup>3</sup> We speculate that this may, in part, have occurred because of a lack of understanding of the underlying molecular mechanism(s) of latrepirdine. Given our observations reported herein, any disparity in the contribution of  $\alpha$ -syn to the neuropathology in the Russian vs US latrepirdine studies might also explain, at least in part, the inconsistency of the cognitive benefit in the two trials. If this speculation were correct, then one would predict that latrepirdine might be more beneficial in treating synucleinopathies such as PD, Lewy body dementia, REM sleep disorder and/or multiple system atrophy by comparison with AD, in which an unpredictable and lower percentage (30–50%) of the clinical population harbors  $\alpha$ -syn neuropathology.<sup>13–15</sup> As animal models of these diseases are now available, one important direction of this research will be to assess the potential of latrepirdine to improve the neuropathology and/or clinical manifestations of synucleinopathy. These experiments are now underway.

## CONFLICT OF INTEREST

AAP is Vice President of Preclinical Development for Medivation. SG holds research grant support from Amicus Pharmaceuticals and is a consultant to the Pfizer-Janssen Alzheimer's Immunotherapy Alliance. GAP is on the scientific advisory boards of Amicus Pharmaceuticals and Neurophage. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)