Pharm Res (2018) 35: 49
https://doi.org/10.1007/s11095-017-2312-2

RESEARCH PAPER

Dihydroceramide Desaturase 1 Inhibitors Reduce Amyloid-β Levels in Primary Neurons from an Alzheimer's Disease Transgenic Model

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Received: 10 July 2017 / Accepted: 16 November 2017 / Published online: 6 February 2018

ABSTRACT

Purpose The induction of autophagy has recently been explored as a promising therapeutic strategy to combat Alzheimer's disease. Among many other factors, there is evidence that ceramides/dihydroceramides act as mediators of autophagy, although the exact mechanisms underlying such effects are poorly understood. Here, we describe how two dihydroceramide desaturase inhibitors (XM461 and XM462) trigger autophagy and reduce amyloid secretion by neurons.

Methods Neurons isolated from wild-type and APP/PS1 transgenic mice were exposed to the two dihydroceramide desaturase inhibitors to assess their effect on these cell's protein and lipid profiles.

Results Both dihydroceramide desaturase inhibitors increased the autophagic vesicles in wild-type neurons, reflected as an increase in LC3-II, and this was correlated with the accumulation of dihydroceramides and dihydrosphingomyelins. Exposing APP/PS1 transgenic neurons to these inhibitors also produced a 50% reduction in amyloid secretion and/or production. The lipidomic defects triggered by these dihydroceramide desaturase inhibitors were correlated with a loss of S6K activity, witnessed by the changes in S6 phosphorylation, which strongly suggested a reduction of mTORC1 activity.

Conclusions The data obtained strongly suggest that dihydroceramide desaturase 1 activity may modulate autophagy and mTORC1 activity in neurons, inhibiting amyloid secretion and S6K activity. As such, it is tantalizing to propose that dihydroceramide desaturase 1 may be an important therapeutic target to combat amyloidosis.

KEY WORDS alzheimer's disease · amyloid-β · APP/PS1 · autophagy · dihydroceramide desaturase 1

ABBREVIATIONS

Aβ	Amyloid β peptide
AD	Alzheimer's disease
APP	Amyloid precursor protein
BafA1	Bafilomycin A
Cer	Ceramides
Des1	Dihydroceramide desaturase 1
Des2	Dihydroceramide desaturase 2
dhCer	Dihydroceramides
FAD	Familial alzheimer's disease
LC3	Microtubule-associated protein 1A/1B–light chain 3
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
p62	Nucleoporin p62
PCR	Polymerase chain reaction
PS1	Presenilin 1
RV	Resveratrol
SLs	Sphingolipids
TGN	Trans golgi network

Guest Editor: Davide Brambilla

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11095-017-2312-2) contains supplementary material, which is available to authorized users.

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INTRODUCTION

Alzheimer's disease (AD) is the most prevalent type of dementia, and it is characterised by neurodegeneration, neurofibrillary tangles and beta-amyloid deposits (1,2). Most AD cases (90–95%) are *sporadic* (of unknown cause) (3), yet a small proportion of AD is of genetic origin, known as Familial Alzheimer Disease (FAD), and this has been related to certain mutations. The accumulation of different proteins in certain neurodegenerative diseases (i.e.: AD, Parkinson's, Huntington's etc.) or *proteinopathies*, has led to the proposal that a defect in proteostasis is a common feature of these pathologies, affecting either the proteasome or mechanisms of autophagy (4,5). Autophagy is a cellular catabolic pathway that involves lysosomal degradation and the recycling of organelles, intracellular pathogens and proteins (6,7). This pathway is activated after stress as a protective mechanism to obtain nutrients and energy (8). Defects in autophagy are linked to several pathologies, not only neurodegenerative but also heart diseases, altered immune responses, aging and cancer (9). Indeed, aberrant autophagy has been associated with defects in the clearance of harmful amyloid-β (Aβ) aggregates and the Aβ load in neuronal cells decreases when the autophagic flux is enhanced (10). The presence of abnormal autophagic activity and or autophagic markers is frequently observed in the brain of patients with neurodegenerative diseases (11,12). Indeed, autophagy has been proposed to play a critical role in the pathogenesis of AD, either in the initial steps and/or in its progression, and the activation of autophagy may dampen Aβ and Tau pathologies, subsequently improving learning and memory (13,14).

Sphingolipids (SLs) are lipids that are ubiquitous in the eukaryotic membrane and they participate in the generation of various membrane structures (e.g., lipid rafts), thereby influencing a number of activities in the cell including autophagy (15). In addition, the SLs could act as important regulators of both apoptosis and autophagy (16). In fact, the SL metabolites, dihydroceramide (dhCer), ceramide (Cer), sphingosine-1-phosphate and gangliosides are emerging as important signalling molecules that contribute to the pathogenesis of inflammatory diseases (17). Dihydroceramide is the immediate metabolic precursor of ceramide in *de novo* sphingolipid synthesis and it is converted into ceramide by the action of dihydroceramide desaturases (*Des 1* and *Des 2*) (18).

Des1 is associated with an electronic transport chain that includes NADPH-cytochrome b5 reductase and cytochrome b5, and it is active at the cytosolic face of the endoplasmic reticulum. When Des 1 is silenced, cells exhibit high levels of autophagy, in part due to impaired ATP synthesis that leads to the activation of AMP-activated protein kinase (19).

To the best of our knowledge, there is no direct evidence of an association between Des1 and AD. Indeed, the only indirect evidence in support of Des1 as a target for AD comes from studies of the effects of some drugs and nutritional factors that may inhibit Des1 activity. For instance, it has been seen that resveratrol (RV) or tocotrienols (vitamin E components) may affect amyloidosis in animal models (20–22). Resveratrol appears to induce autophagy in gastric cancer cells after an increase in dihydroceramides but not ceramides, and it also inhibits dihydroceramide desaturase, inducing effects comparable to those exerted by the dihydroceramide desaturase inhibitor XM462 (18,23).

Here we have examined the effect of inhibiting Des 1 in primary neurons from a double transgenic mouse model expressing human mutations in Amyloid Precursor Protein (APP) and Presenilin 1 (PS1) (24). The neurons of these transgenic mice accumulate Aβ as diffuse neuritic plaques in an age dependent manner, and they display some dysfunctional neuronal proteins (25). We show that the Des1 inhibitors XM461 and XM462 induce autophagy in wild-type and transgenic neurons, effects that are reflected by modifications of the lipid profile of these neurons, reducing the amount of ceramide, sphingomyelin and glucosylceramide, while increasing their dihydro counterparts (i.e.: dihydroceramide, dihydrosphingomyelin, etc.). These changes in lipids are correlated with an increase in autophagic markers. Finally, as a consequence of these events there is a significant reduction of amyloid secretion in the neurons isolated from transgenic APP/PS1 mice.

MATERIALS AND METHODS

Materials

Resveratrol was purchased from Cayman Chemical Company (USA), XM462 was synthesized as reported previously (23) and XM461 was prepared following the same procedure but using pivaloyl chloride in the *N*-acylation step.

Culture of Primary Cerebellar Granule Cells

The dual transgenic B6.Cg-Tg (APPSwe, PSEN1dε9) mice (Jackson Laboratory, Bar Harbor: stock no. 005864) were used to obtain neurons that produce Aβ. All animal care and handling was in accord with current Spanish legislation and guidelines as well as those by the European Commission (directive 2010/63/EU). Neurons from the transgenic mice and their wild-type littermates were used to analyse the effect of compounds on normal neurons and those that produce Aβ. We isolated granule cerebellar neurons from the cerebellum of 5 to 7 days-old transgenic and wild-type pups, as reported elsewhere (26,27). The meninges were carefully removed from the cerebellum in HBSS/BSA medium, and the washed tissue was then minced with a razor blade and digested in trypsin for 15 min at 37°C. The digestion was stopped by adding a HBSS/FBS/DNase solution and a single cell suspension was obtained by triturating the tissue with cotton plugged sterile Pasteur pipettes. After centrifuging to recover the cells, they

were seeded in B27/Neurobasal medium on plates previously coated with poly-L-lysine (Sigma) and then incubated at 37°C in an atmosphere of 5% CO_2 for 48 h until being incubated with the appropriated compound.

Treatments

XM 461 (8 μM), XM 462 (8 μM) and resveratrol (80 μM) were added to the cultured neurons diluted in B27/Neurobasal medium, and the cells were maintained for 24 or 48 h at 37°C and in 5% CO_2. Resveratrol was considered as a positive control compound as it has already been reported to induce autophagy (28,29). The highest concentration of DMSO alone was used as a negative control.

To assay cytotoxicity and to measure extracellular Aβ, cells were seeded in 24 well plates (Falcon) at a density of 10^5 cells/well in 500 μl of B27/Neurobasal medium. At least three independent experiments were performed and 3 or 4 replicate wells were employed for each concentration tested. After the appropriate exposure (24 or 48 h), 200 μl of the medium was taken from each well and centrifuged for 5 min at 13,000 rpm to eliminate floating cells and detritus. The supernatants were then stored at −20°C before Aβ levels were measured by ELISA. The cells cultured in the plates were used to measure the cytotoxic effects of the compounds. Biochemical analyses were performed on 10^6 cells/well plated in 6 well-plates (Falcon) and maintained in 2 ml of B27/Neurobasal medium. After treatment, the cells were washed with PBS and immediately frozen on dry-ice.

Cytotoxicity

Cell viability was examined by means of the MTT assay, which depends on the reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble formazan by living cells (30). After treatment, the cells were exposed for 1 h at 37°C to an MTT solution (0.5 mg/mL) diluted in phenol free Neurobasal medium. The MTT solution was then removed and DMSO was added to the cells. The supernatant was transferred to a 96-well plate and its absorbance was measured at 540 nm using an Opsys MR microplate reader (Dynex Technologies).

Cell number was determined using a Coomassie Blue Assay (31), first fixing the cells with 4% paraformaldehyde (PFA) and then staining them with Coomassie Blue R-250. Intracellular staining was extracted with 0.1 N NaOH and dissolved in 10% trichloroacetic acid (TCA). The absorbance was measured at 595 nm on a microplate reader.

Autophagic Flux

The level of autophagic flux was measured by blocking fusion with lysosomes and the clearance of autophagosomes by adding the v-ATPase inhibitor Bafilomycin A1 (BafA1: Santa Cruz Biotechnology). Accordingly, BafA1, or an equal volume of DMSO, was added to the neurons in culture at a final concentration of 100 nM for the last 2 h of treatment before harvesting the cells for western blotting.

Gel Electrophoresis and Western Blots

Treated cells were scraped off the plates in 50 μl of ice-cold lysis buffer (20 mM Hepes, 100 mM NaCl, 100 mM NaF, 1 mM $NaVO_4$, 5 mM EDTA, 1% Triton X100) containing a protease inhibitor cocktail (Roche Diagnostic) and the phosphatase inhibitor okadaic acid (1 μM: Calbiochem). The homogenate was centrifuged at 16,000×*g* for 20 min at 4°C and the supernatant was stored at −80°C. The protein concentration was measured using the BioRad DC Protein Assay (BioRad), following the manufacturer protocol. Prior to resolving the proteins (10 or 20 μg protein/lane) by SDS/PAGE (PolyAcrylamide Gel Electrophoresis), they were diluted in loading buffer (10% SDS, 0.5 mM DTT, 325 mM TrisHCl [pH 6.8], 87% glycerol, and bromophenol blue). Proteins were then transferred to nitrocellulose (Whatman) or PVDF (Millipore) membranes and after blocking in a 10% solution of non-fat milk, the membranes were probed overnight at 4°C with the primary antibody (Table I). After washing in 0.1% Tween-PBS, the membranes were incubated with the secondary horseradish peroxidise-conjugated antibody (Santa Cruz Biotechnology) and antibody binding was detected using Western lighting™ Plus ECL (Perkin-Elmer), with β-actin as the internal control. The intensity of each band was determined on a GS-800™ Calibrated Densitometer (BioRad) using the Image Lab® software (Bio-Rad).

Dihydroceramide Desaturase Activity

The pelleted cells were lysed by sonication and dihydroceramide desaturase activity was determined as reported elsewhere (23). Briefly, after the treatments the cells were lysed and incubated for 4 h at 37°C with a fluorescent substrate (10 μM) in phosphate buffer in the presence of NADH (125 μM). The reaction was then stopped by adding 700 μl methanol and storing the lysates at 4°C overnight. The resulting suspensions were centrifuged for 3 min at 10,000 rpm and the supernatant was analysed by HPLC under the following conditions: mobile phase, A (H_2O + 0.1% TFA) 15%: B (Acetronitile +0.1% TFA) 85%; a Kromasil column 100 C18 5u 100A; Alliance fluorescence detector (excitation 470 nm, emission 530 nm); flow rate, 1 ml/min; and injected volume, 10 μl.

Lipid Analysis

After a 48 h treatment, the neurons were washed 3 times with PBS and frozen at −80°C until analysis. The lipid analysis

Table I List of the Antibodies Used to Probe Western Blots and for Immunofluorescence

Antigen	Host	References
phospho-Synapsin (Ser9)	Rabbit	#2311 Cell Signaling
p120	Mouse	#610133 BD Transduction Laboratories™, USA
synaptophysin	Rabbit	#ab23754 Abcam
Amyloid-β, 1–16 (6E10)	Mouse	#SIG-39320 Covance, California, USA.
phospho-S6 (Ser240/244)	Rabbit	#2215 Cell Signaling, USA
p62	Mouse	#H08878-M01 Abnova-novus biologicals, USA
LC3B	Rabbit	#L7543 Sigma-Aldrich®
β-actin	Mouse	#A5441 clone AC-15 Sigma
GAPDH	Rabbit	#2118 Cell Signaling

could not be performed on neurons exposed to XM462 due to the toxicity of this compound, which impaired the recovery of sufficient material. Otherwise, lipid profiles were analysed as indicated previously (18).

ELISA Quantification of Aβ1–40

The Aβ levels in the medium of the cultured neurons were measured using the Human Aβ40 ELISA kit (Invitrogen), according to the manufacturer's instructions, and measuring the absorbance at 450 nm using an Opsys MR microplate reader (Dynex Technologies).

Statistical Analyses

Two or three way ANOVA tests were performed to check the significance of any differences. In all cases, differences were considered statistically significant when $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

RESULTS

XM461 and XM462 Reduce Des1 Activity

We first investigated whether, as expected from the literature (23), the compounds XM461 and XM462 efficiently diminish Des1 activity in neurons, both those obtained from APP/PS1 mice and their wild-type littermates. These primary neurons were maintained for 48 h in a serum-free medium (NB-B27) and then exposed to the test compounds (8 μM) in the same serum free medium. After 24 and 48 h the cells were recovered and their Des1 activity was determined (see Methods). The two compounds assayed provoked a reduction of around 20% in Des1 activity in both transgenic and wild-type neurons after a 24 h exposure, similar to that provoked by resveratrol that was used as an internal control (Fig. 1a). Furthermore, the inhibition of Des1 after 48 h was more evident in all cases, reaching a reduction of nearly 50% (Fig. 1b).

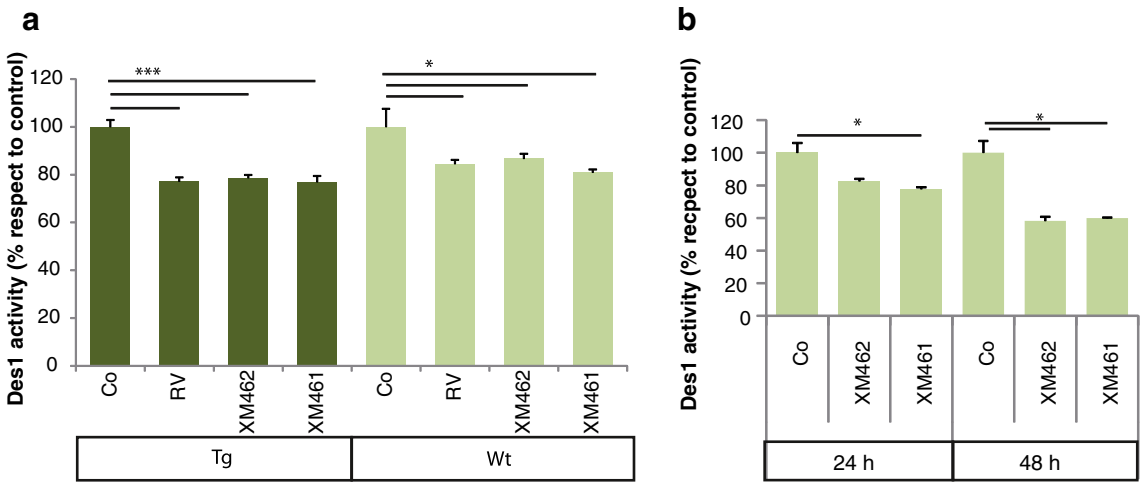

Fig. 1 Dihydroceramide desaturase activity. Neurons from transgenic (Tg) or wild-type (Wt) littermates were treated with resveratrol (RV), XM461 or XM462 48 h after plating; DMSO was used as control (Co). After 24 and 48 h cell extract was obtained and dihydroceramide desaturase activity was determined as described in Methods. (**a**) The quantitative data shows a reduction of Des1 activity caused by resveratrol, XM461 and XM462 inhibitors in transgenic and wild-type neurons. (**b**) Reduction of Des1 activity caused by XM461 and XM462 inhibitors is more evident after 48 h. The graphs represent the percentage respect to control levels (100%) as the medium ± SEM and statistical significance obtained by ANOVA is showed as *$p < 0.05$ or ***$p < 0.001$.

Effect of XM461 and XM462 on Cell Viability

In some tumour cells the inhibition of Des1 has been correlated with enhanced cell death (32,33) or cell cycle arrest/delay (19,34). The effect of XM461 and XM462 on neurons viability was determined by counting the Coomassie Blue stained cells and in parallel, with a standard MTT assay of cell viability. To determine the putative toxicity of these compounds, neurons were incubated for 24 and 48 h with resveratrol, XM461 and XM462 diluted in NB-B27 medium. After exposure to these compounds, a Coomassie Blue assay was used to quantify protein concentrations as an indirect measure of the number of cells given that neurons are post-mitotic cells. While the wild-type neurons were not affected by any compound after 24 or 48 h (Fig. 2a), there was a slight reduction (11%) in the protein quantified in transgenic neurons after exposure to XM462 for 24 h. Moreover, a more pronounced effect was observed on transgenic neurons for all the treatments after 48 h, with resveratrol, XM462 and XM461 each provoking a significant reduction in protein, reaching 12, 24 and 20% respectively.

Cytotoxicity was evident in the MTT assay, which measures the metabolic activity of NAD(P)H-dependent cellular oxidoreductase enzymes (mostly due to mitochondrial activity). XM461 and XM462 both proved affect mitochondrial metabolism, even after a 24 h exposure, with XM462 proving to be the most toxic compound in agreement with the data from the Coomassie assay. These results were similar in wild-type and transgenic neurons after 24 and 48 h (Fig. 2b). Moreover, while resveratrol did not present any toxic effect after a 24 h exposure, it reduced mitochondrial activity after 48 h, as witnessed in the MTT assay (Fig. 2b).

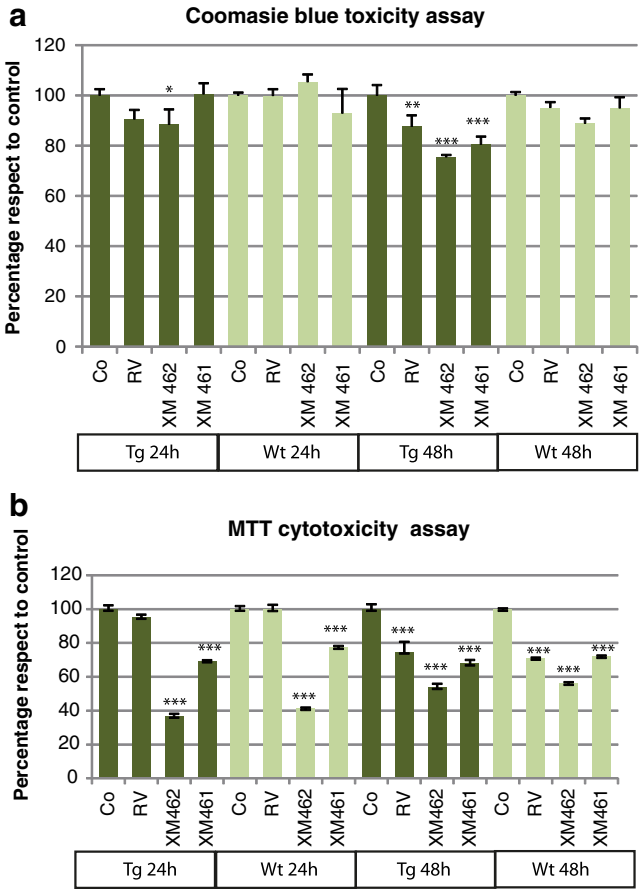

Fig. 2 Coomassie and MTT assays. Neurons from transgenic (Tg) or wild-type (Wt) littermates were treated with resveratrol (RV), XM461 or XM462 48 h after plating; DMSO was used as control (Co). After 24 and 48 h cell viability was determined by two methods: coomassie and MTT. (**a**) Fixed cells after different treatments were stained with Coomassie as described in Methods. The level of staining was determined and the data was represented as the percentage of absorbance observed respect to control cells (as 100%). (**b**) In parallel cultures, after 24 or 48 h of treatment the neurons were incubated with MTT solution for 1 h at 37°C. The purple formazan produced by neurons was extracted and quantified. The data was represented as the percentage of absorbance respect to Wt-control neurons (100%), and analysed using ANOVA test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Effect of Des1 Inhibitors on Neuronal Proteins

After observing some side-effects on neurons, we assessed whether the compounds affected some synaptic markers and axonal proteins, in these neurons, in particular the presynaptic proteins p120, phospho-synapsin and synaptophysin. These proteins are common markers of synaptic activity. First, we confirm the reported differences between Tg and Wt mice showing statistically significant differences in phospho-synapsin and synaptophysin. However, after 24 h, neither p120 nor phospho-synapsin were affected by these Des1 inhibitors in wild-type neurons. Only synaptophysin was modified by XM462 in transgenic neurons after a 24 h exposure. Nevertheless, longer exposures (48 h) produced some modifications in the accumulation of these synaptic markers. In particular, XM462 produced a significant reduction (more than 50%) in all three markers studied in both wild-type and transgenic neurons, probably reflecting the effects of this compound on neuronal viability (Fig. 3). By contrast, XM461 did not significantly affect phospho-synapsin and while there appeared to be a reduction in p120 in both transgenic and wild-type neurons, this was only significant in wild-type cells. Similarly, whereas XM461 did not affect the synaptophysin levels in transgenic neurons, in wild-type neurons synaptophysin levels fell by approximately 50% relative to the controls (Fig. 3).

In parallel, we analysed whether the compounds affected the amyloid precursor protein (APP) in transgenic and wild-type neurons. As expected, there was a clear difference in the APP detected in transgenic and wild-type neurons in western blots, confirming the genotypes. There were no alterations to APP after a 24 h exposure to any of the compounds, whereas

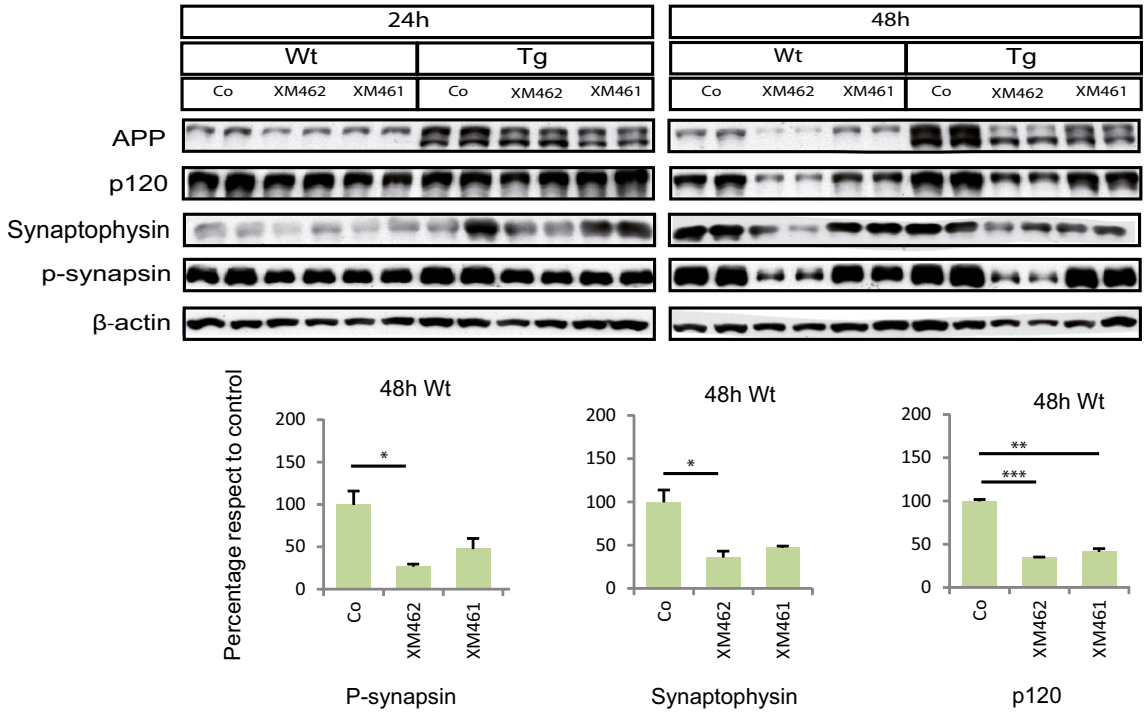

Fig. 3 Neuronal Proteins. Neurons from transgenic (Tg) or wild-type (Wt) littermates were treated with XM461 or XM462, and DMSO was used as control (Co). After 24 or 48 h, cell extracts were obtained and analysed by western-blot using antibodies against APP, p120, Synapsin and Synaptophysin. The quantitative data were represented as the percentage of absorbance respect to control Wt-neurons (100%) using ANOVA test (*$p < 0.05$, **$p < 0.01$).

after 48 h only XM462 reduced APP levels in both transgenic and wild-type neurons, again probably reflecting its effect on viability. Moreover, XM461 only slightly affected the APP levels in the transgenic neurons.

Des1 Inhibition Alters the Sphingolipid Profile of Primary Neurons

Considering the negative effects of XM462 on cell viability after an extended exposure, only the effect of XM461 on lipids was analysed, evaluating its effects on ceramides, dihydroceramides, sphingomyelins, dihydrosphingomyelins and glucosylceramides of acyl chain length from C14 to C24 (Figs. 4 and S1). This analysis showed that exposure of wild-type and transgenic neurons to either resveratrol or XM461 for 48 h modified their lipid profile (Fig. 4). There appeared to be significantly different levels of ceramides and sphingomyelins between untreated wild-type and transgenic neurons ($p < 0.001$ and $p = 0.004$, respectively), whereas there were no differences in dihydroceramides, dihydrosphingomyelins and glucosylceramides between these genotypes. Treatment with XM461 produced an important increase in the dihydroceramides and dihydrosphingomyelins relative to the control or resveratrol treated neurons ($p < 0.001$). In parallel, treatment with both resveratrol and XM461 lowered the levels of ceramides and sphingomyelins particularly in the neurons exposed to resveratrol (Fig. 4). Finally, these data were almost undistinguishable if only the main component (C18) was considered (Fig. 4, left lanes) or if the sum of the C14 to C24 components for each component were analysed (Fig. 4, right lanes).

Des1 Inhibition Induces Autophagy

It has been demonstrated that inhibiting Des1 activates autophagy in several cell types (18) and we found that Des 1 inhibitors increased LC3-II levels in neurons (Fig. 5). The levels of LC3-II increased in transgenic and wild-type neurons treated with resveratrol, XM461 or XM462 for 24 h. To analyse whether these Des1 inhibitors may affect mTORC1 activity, a main regulator of autophagy, we used the S6K kinase activity as a reporter. Thus, we analysed the S6K activity through phospho-S6 and we observed that XM461 and XM462 and at lesser extent RV, reduced phospho-S6 levels.

Autophagy affection was more evident in wild-type neurons treated with XM461 and XM462, and the inhibitor XM461 maintained this effect after 48 h (the cytotoxicity elicited by XM462 impeded assessing autophagy after 48 h). At this time, the phosphorylation levels of phospho-S6 were

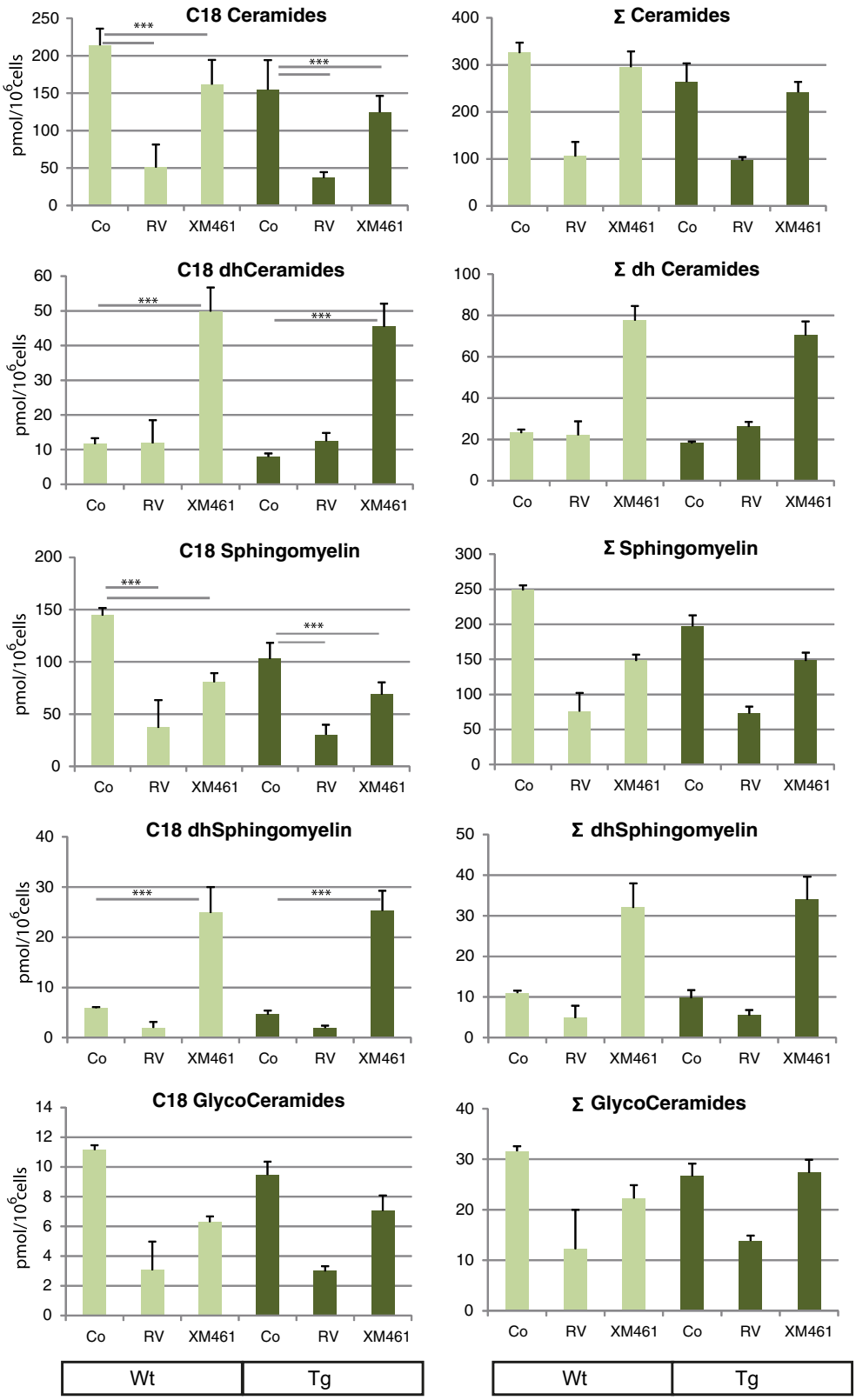

Fig. 4 Lipid profile in wild-type and transgenic neurons. Neurons from transgenic (Tg) or wild-type (Wt) littermates were treated with XM461, resveratrol (RV) or DMSO, used as control (Co). After 48 h, neurons were processed as described in Methods. The levels of ceramides, dihydroceramides, sphingomyelin, dihydrosphingomyelin and glucoceramides with 18 carbons (C18) were quantified. In parallel, the summation of all the analysed species from C14 to C24 is shown (detailed of each population in Figure S1). ***$p < 0.001$.

almost undetectable both in RV or XM461 treated neurons. These data permit to hypothesize that the activation of S6K is almost completely inhibited. The marker p62 showed a clear reduction after 48 h in the presence of XM461, but this effect was not so evident in the presence of RV. The levels of p62 in wild-type and transgenic exposed to RV or XM461 were

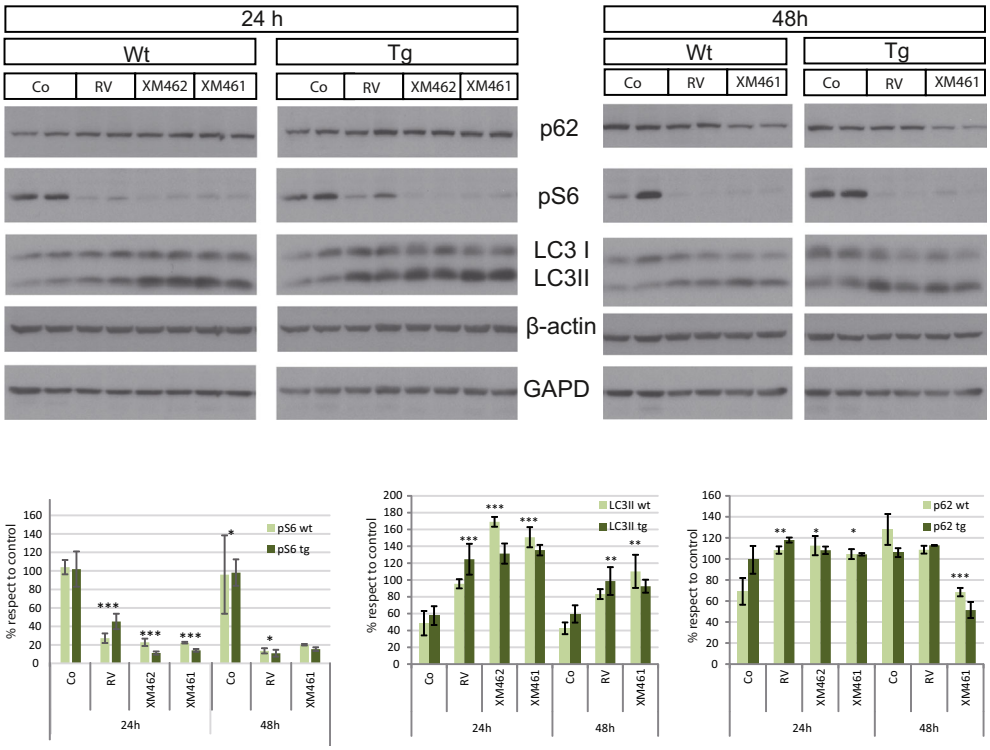

Fig. 5 Analysis of autophagic markers. Neurons from transgenic (Tg) or wild-type (Wt) littermates were treated with resveratrol (RV), XM461 or XM462 and DMSO, used as control (Co). After 24 and 48 h, cell extract was obtained and analysed by western-blots using antibodies against LC3 and p62 as autophagic markers, pS6 as reporter of S6K activity and **β-**actin and GAPDH as loading controls. The quantitative data was represented as the percentage of absorbance respect to control neurons (the data from Wt neurons was arbitrarily consider as 100%) and analysed using three-way ANOVA test. * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$.

similar. All together, these data strongly suggest that XM461 and XM462 enhanced autophagy.

Then, we performed a new experiment to analyse the autophagic flux, for which BafA1 was added for the last 2 h of treatment (Fig. 6). The amount of LC3-II and p62 immunoreactivity increased after a 2 h exposure to Bafilomycin relative to the untreated controls. This increase was even more evident in neurons treated with XM461 or XM462 than with RV. The differences in LC3-II were maintained after 24 h of treatment, whereas the lower p62 levels after a 24 h treatment with XM461 or XM462 highlighted the efficient degradation of this long-lived protein. By contrast, the levels of p62 in resveratrol treated neurons were similar to those in control untreated neurons (Fig. 6). The higher autophagic flux reflected by the LC3-II levels and the increase in the degradation rate indicated by the autophagic adaptor p62, led us to conclude that both XM462 and XM461 are efficient inducers of autophagy in cultured neurons. In contrast RV in wild type neurons had a low autophagic flux and low degradation rate of p62.

Inhibition of Des 1 Reduced the Levels of Amyloid

Some compounds that induce autophagy have been demonstrated to reduce amyloid-β levels *in vivo* or *ex vivo* (in culture) and thus, we checked the effect of XM461 and XM462 on the amyloid produced by transgenic neurons. In these assays we used RV as a control as it is reported to induce autophagy, as confirmed by some of our previous data. No modification in amyloid levels was evident after 24 h (Fig. 7) and neither XM461 nor XM462 significantly lowered the secretion of this peptide. It should be noted that the medium was totally removed when the experimental compounds were added and thus, we are analysing the effect on newly secreted amyloid production. However, after 48 h, the presence of RV reduced amyloid production by nearly 30% with respect to controls. Interestingly, XM461 reduce amyloid levels to 50% that of the controls, whereas the effect of XM462 treatment was more modest and it produced only a 30% reduction (Fig. 7).

DISCUSSION

This study was undertaken to test the effect of Des 1 inhibitors on the induction of autophagy and on amyloid-β production and/or accumulation. Previous reports support a role of SL metabolism in autophagy (35–37) and similarly, studies using pharmacological inhibitors that reduce Des1 activity (38) or those on fibroblasts from a DES1 gene knock-out mice (19) provided further support of a role for dhCer in inducing autophagy. Increased dhCer levels have been proposed to drive increased autophagic flux (39), and we previously found that Des1 inhibitors like XM462 can induce autophagy through dhCer dependent and independent pathways (18). Importantly, the activation of autophagy, for instance by exposing cells to rapamycin or when activated *in vivo*, provoked a significant reduction in amyloid secretion (13,14,40). Here, we analysed the autophagic effect of XM462 and a new Des1

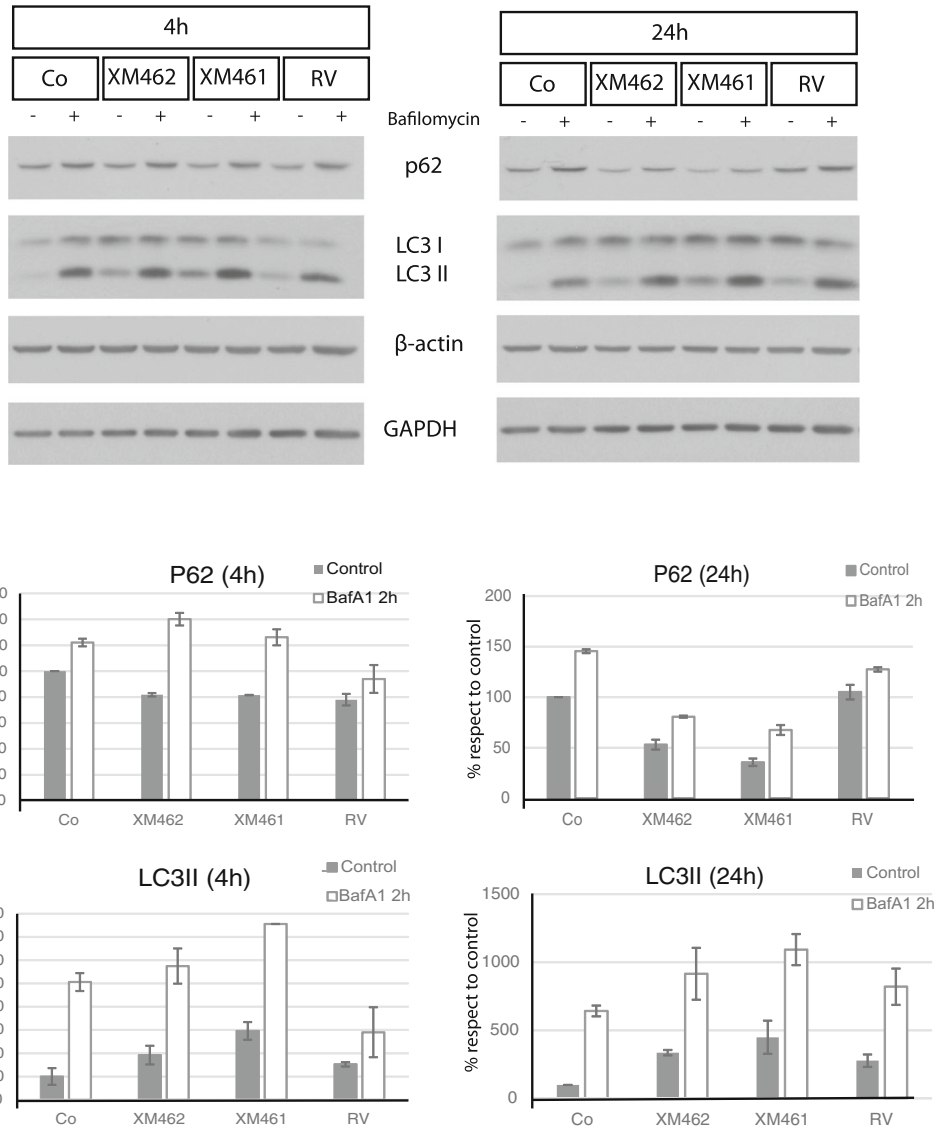

Fig. 6 Autophagy flux determination. Neurons from transgenic (Tg) or wild-type (Wt) littermates were treated with resveratrol (RV), XM461 or XM462, using DMSO as control (Co). The treatment was maintained for either 4 or 24 h. In a parallel experiment bafilomycinA1 (BafA1) was added for 2 h to a complete set of identical conditions. Then cell extracts were analysed by western-blot using antibodies against LC3, p62, **β-**actin and GAPDH (as loading controls). The quantitative data was represented as the percentage of absorbance respect to control neurons (considered as 100%).

inhibitor, XM461, in primary cultured neurons from APP/PS1 transgenic mice and their wild-type littermates. We demonstrate that dampening Des1 activity with either inhibitor augmented the autophagic flux markers in these cells (monitored by LC3-II and p62), and at both times (24 and 48 h). Moreover, both compounds produced a decrease in amyloid secretion. By contrast, RV showed only a tendency to enhance autophagy in wild-type neurons when considering LC3-II, in experiments with or without Bafilomycin, as described previously (20,38). It is not so evident when considering p62 degradation rate. Indeed, in contrast to Des-1 inhibitors, RV actually reduced the ceramides and barely increased dh-ceramides. All these data permit to conclude that Des1 inhibitors and resveratrol modify different aspects of sphingolipid metabolism affecting in different ways autophagy.

The second feature that we analysed was the capacity of these inhibitors to trigger autophagy reducing mTORC1 activity. Certainly, the pS6 reduction strongly suggested that both inhibitors affect similarly to S6K activity, which permit to infer that both reduce the kinase activity of mTORC1.

It is important to remember two main issues. Firstly, the inhibition of Des1, the last enzyme in the *de novo* biosynthesis of ceramide, provokes an increase in the dihydrosphingolipids in lipid rafts and it would impair biological processes linked to these microdomains (41). Secondly, Aβ peptides are generated through the sequential proteolytic cleavage of APP by specific secretases (β- and γ-Secretase) at lipid rafts in neuronal cell membranes (42,43). The amyloidogenic process is triggered by β-Secretase and APP, both transmembrane proteins that traffic from the Golgi/trans Golgi network (TGN) to the extracellular membrane. β-secretase is the initial and rate-limiting enzyme of this process (44–46) and there is data suggesting that only after endocytosis is APP processed by β-secretase (BACE) and then by PS1/2 to generate different

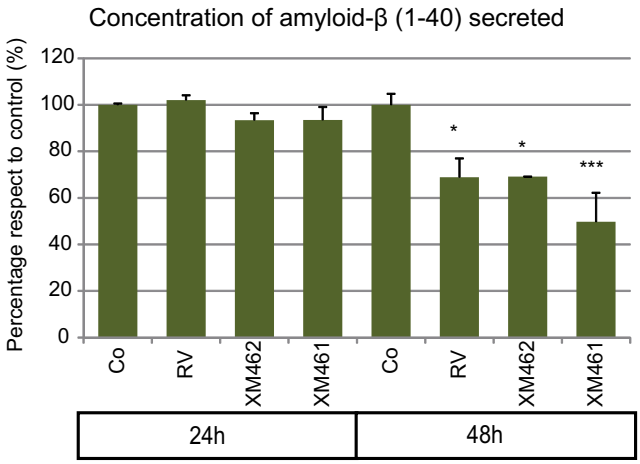

Fig. 7 Des1 inhibitors impaired Aβ-peptide (1–40) production. Neurons from transgenic pups were maintained for 48 h in defined medium (NB-B27). Then, the medium was replaced and added resveratrol (RV), XM461, XM462 or DMSO as control (Co). After 24 and 48 h cell culture medium was taken to measure the concentration of amyloid-β peptide excreted by the neurons. The data was determined and represented as the percentage respect to control group (100%) and analysed by ANOVA test (* $p < 0.05$, *** $p < 0.001$).

Aβ peptides (1–40/42/43) (2,47). In fact, alterations in endosome-lysosome trafficking and Aβ accumulation within endosomes have been described as the earliest findings in AD brains (12,48). Moreover, multivesicular bodies and late endosomes containing Aβ42 are present in synaptic terminals of mutant APP transgenic mice (49). Besides, defects in the clearance of Aβ aggregates have been associated with aberrant autophagy and it has been shown that the Aβ load in neuronal cells decreases by increasing the autophagic flux (10).

All these data strongly suggest that modification of endosome-lysosome degradation is impaired in the AD brain, in clear correlation with impaired autophagy. Considering that the cleavage of APP depends on the correct physical interaction between APP and BACE, modification of the properties/composition of the membrane may be an important event that could positively or negatively affect this interaction. In this sense, blocking essential enzymes that control the synthesis of lipids may be a novel strategy that could alter Aβ generation/degradation. Our data show that unlike resveratrol, Des1 inhibitors substantially modify the ratio of some dihydrosphingolipids relative to their unsaturated counterparts. Indeed, ceramide has been implicated in the induction of autophagy in various models (50). The accumulation of dhCer apparently enhances cell survival during hypoxia by inducing autophagy, whereas it may also serve as a reservoir for the rapid production of ceramide to combat cell damage upon reperfusion (51).

We certainly don't know whether the change in lipid repertoire may be responsible for the neuronal proteins modifications. While in the case of the inhibitor XM462 may be attributed to the toxic effect evident after 48 h. Moreover, the inhibitor XM461 does not appear to affect neuron viability yet it produced a clear modification of p-Synapsin or Synaptophysin. Obviously, more work has to be done to clarify the connection between ceramides content and these proteins modifications. The mechanisms linking the modification of lipid profiles and autophagy are unknown; however, our data show that Des1 inhibitors trigger an important reduction of S6K activity. This can be inferred by the accumulation of pS6, which resembles the effects of rapamycin inhibition of mTORC1, that would be, directly or indirectly a potential mediator.

CONCLUSION

From the data obtained, we hypothesize that by altering the biophysical properties of lipid rafts in the membranes of neurons, inhibiting Des1 will produce two important alterations. First, it would reduce beta-secretase activity or reduce the membrane domains containing APP and BACE, impeding amyloidogenic processing of APP. Secondly; it will enhance the autophagic flux, which will lead to the generation of less Aβ peptide. Together, these data permit us to postulate that Des 1 is a new target for amyloidosis and that Des 1 inhibitors like XM461 could represent a neuroprotective agent in proteinopathies like AD, due to its ability to enhance autophagy.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by a grant from the Centro de Investigacion Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED; an initiative of the ISCIII). In addition, work in FW's lab was supported by grants from the "Plan Nacional", "Dirección General de Ciencia y Tecnología - DGCYT SAF2012-39148-C03-01; and Proyectos I+D+i Retos 2015 SAF2015-70368-R, and an Institutional grant from the" Fundación Areces".

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