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## Alzheimer's Disease Presenilin-1 Mutation Sensitizes Neurons to Impaired Autophagy Flux and Propofol Neurotoxicity: Role of Calcium Dysregulation

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### Abstract

**Background:** Disruption of intracellular Ca<sup>2+</sup> homeostasis and associated autophagy dysfunction contribute to neuropathology in Alzheimer's disease (AD).

**Objective:** To study the effects of propofol on cell viability via its effects on intracellular Ca<sup>2+</sup> homeostasis, and the impact of autophagy, in a neuronal model of presenilin-mutated familial AD (FAD).

**Methods:** We treated PC12 cells, stably transfected with either mutated presenilin-1 (L286V) or wild type (WT) controls, with propofol at different doses and durations, in the presence or absence of extracellular Ca<sup>2+</sup>, antagonists of inositol trisphosphate receptors (InsP<sub>3</sub>R, xestospongine C) and/or ryanodine receptors (RYR, dantrolene), or an inhibitor of autophagy flux (Bafilomycin). We determined cell viability, cytosolic Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>c</sub>), vATPase protein expression, and lysosomal acidification.

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**Results:** The propofol dose- and time-dependently decreased cell viability significantly more in L286V than WT cells, especially at the pharmacological dose ( $>50 \mu\text{M}$ ), and together with bafilomycin (40 nM). Clinically used concentrations of propofol ( $<20 \mu\text{M}$ ) tended to increase cell viability. Propofol significantly increased  $[\text{Ca}^{2+}]_c$  more in L286V than in WT cells, which was associated with decrease of vATPase expression and localization to the lysosome. Both toxicity and increased  $\text{Ca}^{2+}$  levels were ameliorated by inhibiting  $\text{InsP}_3\text{R}/\text{R/YR}$ . However, the combined inhibition of both receptors paradoxically increased  $[\text{Ca}^{2+}]_c$ , by inducing  $\text{Ca}^{2+}$  influx from the extracellular space, causing greater cytotoxicity.

**Conclusion:** Impairment in autophagy function acts to deteriorate cell death induced by propofol in FAD neuronal cells. Cell death is ameliorated by either RYR or  $\text{InsP}_3\text{R}$  antagonists on their own, but not when both are co-administered.

### Keywords

Alzheimer's disease; anesthesia; autophagy; calcium; presenilin

## INTRODUCTION

One of the proposed mechanisms for neuropathology in Alzheimer's disease (AD) is the impairment of autophagy function [1]. This may result in promoting amyloid or tau [2, 3] pathology, increased neurodegeneration [4], and cognitive dysfunction [5].  $\text{H}^+/\text{Ca}^{2+}$  homeostasis in the lysosome plays an essential role in the regulation of autophagy via autophagy gene transcription, mediated by the transcription factor EB [6, 7]. Recent studies suggested that the mutation of AD's presenilin 1 (PS1) has abnormally increased  $\text{Ca}^{2+}$  release from the endoplasmic reticulum (ER) via over activation of either inositol trisphosphate receptors ( $\text{InsP}_3\text{Rs}$ ) or ryanodine receptors (RYRs) and has disrupted lysosomal  $\text{H}^+/\text{Ca}^{2+}$  homeostasis. This disruption has led to impaired lysosome and autophagy function [8–10]. The PS2 mutation has also changed the dynamics of ER and mitochondria tethering and the subsequent  $\text{Ca}^{2+}$  transfer from the ER to mitochondria, an important process for both cell physiological function and pathological damage [11].

General anesthetics may worsen cognitive function in aged rodents [12, 13]. Through examining various tissue cultures and animal models [14], it is clear that general anesthetics worsen amyloid and tau pathology, neurodegeneration, and cognitive dysfunction in AD although the mechanisms remain unclear. Our previous studies suggested that isoflurane activated  $\text{InsP}_3\text{Rs}$  and dysregulated the calcium homeostasis in the ER and the mitochondria and cytosolic space [15, 16], causing cell death. Conversely, over activation of RYRs also contributed to general anesthetics mediated neurotoxicity [17].

General anesthetics also affect the lysosome pH and autophagy function with an unclear molecular mechanism [18]. Herein, we discovered that at pharmacological as opposed to clinically used doses, the commonly used general anesthetic propofol impairs autophagic flux through over activation of  $\text{InsP}_3\text{Rs}$  and/or RYRs, disrupting the synthesis or localization of vATPase, and decreasing lysosome acidity in a cell model of FAD.

## MATERIALS AND METHODS

### Cell cultures

Rat pheochromocytoma cells (PC12) either transfected with wild-type (WT) PS1 or point mutated (L286V) PS1 were utilized to model AD, as L286V cells have been shown to be more vulnerable than WT to various neurotoxin including inhalational anesthetics [19–22]. The cells were maintained in DMEM medium (Life Technologies, Carlsbad, CA, USA) containing 5% fetal calf serum, 10% heat-inactivated horse serum, 200 µg/ml G418 and 1% penicillin/streptomycin [23, 24]. Initial cells were suspended with density of  $0.5 \times 10^5$  cells/cm<sup>2</sup> in culture medium and incubated in T75 flasks in a 95% air, 5% CO<sub>2</sub> humidified atmosphere at 37°C. We changed the medium every 48 h.

### Cell treatment and viability measurement

Cell were treated with various concentrations of propofol (1, 5, 20, 50, 100, 200, 400 µM) for various durations (3, 6, 12 h) as dose- and time-response studies. These concentration and duration are chosen primarily based on the typical clinical range (1–20 µM for less than 6 h) [25] and the high pharmacological range for prolonged use (50–400 µM and for 12 h). Cell viability was measured by using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). One day prior to the experiment, the cells were seeded in a 96-well plate (5000 cells/well) under normal culture condition. Each experiment was repeated for three times to ensure reproducibility. At the end of the various treatments, CCK-8 solution was added to each well at a dilution of 1:10 and the plates were incubated at 37°C for 1 h. Then absorbance measurements at 450 nm were performed by using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA).

### Calcium imaging

Cell were exposed to 200 µM propofol to raise  $[Ca^{2+}]_c$ , which was measured by Fura-2 acetoxymethylester (AM) fluorescence (Invitrogen) on the stage of an inverted microscope (Olympus IX70) and IPLab v3.71 software (Scanalytics, Inc, USA), as previously described [26]. Briefly, the cells were plated onto 35 mm culture dishes for 24 h, washed with Hank's Balanced Salt Solution (HBSS, Sigma H-4891) for three times, and incubated with 2.5 µM Fura-2 AM in the same buffer supplemented with 1% bovine serum albumin (BSA) at 37°C in the dark for 30 min. Then the cells were washed three times with HBSS to remove excess Fura-2 AM. When the cells exposed to various treatment of propofol, in the presence or absence of dantrolene or Xestospongin C, Fura-2 fluorescence intensities were monitored by recording at 340- and 380 nm excitation and at 510 nm emission for up to 10 min. The data are presented as ratios of 340/380 nm of fluorescence intensity normalized to baseline.

### Lysosome acidity measurements

Lysosome acidity was measured by staining with LysoTracker Red DND-99 (Molecular Probe, Eugene, OR), a fluorescent dye for labeling and tracking acidic organelles in live cells. The cell suspensions were plated onto glass coverslips placed in 35 mm plastic culture dishes for 24 h. The next day the culture medium were aspirated, the cells were washed three

times with HBSS containing 1.8 mM CaCl<sub>2</sub> and 0.8 mM MgCl<sub>2</sub> (pH 7.4). The cells were loaded with 50 nM LysoTracker working solution and incubated for 1 h at 37°C. After that, the cells were washed three times with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) for 15 min. We measured the fluorescence intensity of the acidic lysosome organelles at excitation maximum of ~577 nm and emission maximum ~590 nm.

### Immunofluorescence

The cells were seeded onto glass coverslips placed in a six-well culture dishes at 5000 cells/well and allowed to grow for 24 h. Then the cells were rinsed briefly in PBS and fixed with 4% formaldehyde for 10 min at room temperature. After that, the cells were permeabilized with 10% Triton X-100 for 5 min following three washes with PBS and blocked in 5% normal goat/donkey serum for 1 h. Subsequently, the cells were incubated with primary antibodies diluted in blocking buffer (1:50) overnight at 4°C. After overnight incubation, the cells were washed with PBS three times for 5 min each, and followed by a 1 h (at RT) incubation with a secondary antibody in the dark. Lastly, the coverslips were rinsed once in PBS and stained with DAPI for 5 min and mounted on the slides with ProLong Gold Antifade Mountant Medium (Thermo Fisher Scientific, USA).

### Data analysis and statistics

The data is expressed as mean ± SEM and assessed for significance using Student's unpaired *t* test, one-way or two-way ANOVA with Tukey *post hoc* analysis. The significance level for all of our analyses was set at 99% ( $p < 0.01$ ). We used GraphPad Prism software (GraphPad Software Inc.) for all statistical analyses.

## RESULTS

### Autophagy impairment in AD worsened propofol induced neurotoxicity

Previous studies demonstrate that propofol is much less potent than inhalational anesthetics in causing cell damage in various types of cells [27] and in developing brains of rodents [16]. The cells featured with AD were more vulnerable to anesthetics toxicity [28]. Similar to in inhalational anesthetics [29], propofol dose- and time-dependently affected the cell viability in both WT and L286V AD cells (Fig. 1A, B). Propofol at the commonly used clinical concentration (1 μM) trended to increase cell viability in both types of cells determined by CCK assay, while its high end concentration within the clinically used range (20 μM) decreased cell viability in L286V AD cells only when used longer than 6 h, but not in WT cells (Fig. 1A, B). Propofol at clinically relevant concentrations (1–20 μM), rarely induced cell damage, but needed very high non-clinical concentrations (>50–100 μM) to cause cell death [30]. Our results here (Fig. 1A) are consistent with previous studies [31] which indicated that in wild type cells clinically relevant concentrations of propofol did not induce obvious cell damage. Although 20 μM induced injury at 6 h of treatment, treatment for 6 h with up to 400 μM of propofol did not cause more cell damage in L286V cells relative to WT cells (Fig. 1C). In contrast, pretreatment of bafilomycin, an agent that inhibits the lysosomal vATPase and the fusion between the autophagosome and the lysosome [32], significantly increased vulnerability of L286V AD cells to propofol toxicity at higher concentrations (>50 μM, Fig. 1D), suggesting that autophagy impairment, as observed in the

context of PS1 mutations or with aging, exacerbates propofol induced cell damage. Furthermore, propofol mediated cell damage in the presence of bafilomycin could be inhibited by either Xc or dantrolene (Fig. 1E), suggesting a role for calcium dysregulation via InsP<sub>3</sub>Rs or RYRs activation in augmenting propofol-induced cell damage. Paradoxically, however, combined use Xc and dantrolene worsened propofol mediated cytotoxicity (Fig. 1F).

### **Propofol caused abnormal Ca<sup>2+</sup> release from the ER via over activation of InsP<sub>3</sub>Rs or RYRs in AD cells**

Inhalational anesthetics can affect cytosolic Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>c</sub>) via multiple mechanisms. Two such mechanisms include Ca<sup>2+</sup> influx from the extracellular space and Ca<sup>2+</sup> release from the ER [33]. Inhalational anesthetics at high concentrations for prolonged use could induce cell damage via over activation of InsP<sub>3</sub>Rs [15] and/or RYRs [34, 35] in a variety of tissue cultures and in animal models [29, 36, 37]. This may be particularly true in cells features with AD [38] or Huntington disease [37]. Similar to inhalational anesthetics, propofol at a high concentration (200 μM) induced Ca<sup>2+</sup> release from the ER via activation of InsP<sub>3</sub>Rs/RYRs. It also elevated both peak and integrated Ca<sup>2+</sup> response (area under curve, AUC) [Ca<sup>2+</sup>]<sub>c</sub> more significantly in AD than WT PC12 cells, which could be significantly inhibited by either Xc or dantrolene (Fig. 2A, B). Paradoxically, combined use of antagonists for both InsP<sub>3</sub>R and RYR (Xc+dantrolene) did not further decrease propofol-induced elevation of peak and AUC of [Ca<sup>2+</sup>]<sub>c</sub>, as expected, but rather increased it significantly (Fig. 2C, D). This may explain the augmentation of propofol-induced cytotoxicity observed above (Fig. 1F). The paradoxical elevation of [Ca<sup>2+</sup>]<sub>c</sub> in the presence of both inhibitors was dependent upon Ca<sup>2+</sup> influx from the extracellular space because it was significantly inhibited by ethylene glycol tetraacetic acid (EGTA), a calcium chelator added to the extracellular buffer (Fig. 2E, F).

### **PS1 mutation induced defective lysosomal acidification by altered localization and function of the vATPase**

We further explored the vATPase V0a1 subunit as a marker of the proton pump in lysosomes in PS1 mutation cells. Double immunofluorescence labeling analysis demonstrated much stronger colocalization of vATPase with LAMP-2 (lysosome-associated membrane protein-2) in WT cells than that in L286V cells (Fig. 3). Similarly, compared to WT control, acidity in lysosomes determined by LysoTracker in L286V cells also significantly decreased (Fig. 4). Our study is consistent with previous report that the PS1 mutation, like its knock out from cell, significantly decreased acidity in lysosomes by reduced numbers of vATPase. However, propofol at high pharmacological concentration (100 μM) but not at clinically relevant concentration (1 μM) increased LysoTracker staining of acidic compartments, much more dramatically in L286V AD cells, which could be significantly inhibited by either Xc or dantrolene (Fig. 4). Interestingly, although colocalization of vATPase with early endosome antigen-1 (EEA1) was very weak in early endosomes of both WT and L286V cells, it was significantly increased in both types of cells after the treatment of high concentration of propofol (100 μM), suggesting a propofol-mediated vATPase detainment in early endosome and associated decrease in lysosome. Despite an increase of the vATPase V0a1 subunit in ER, decreased Ca<sup>2+</sup> concentration in ER may result in a possible block of vATPase assembly

and eliminated vATPase-dependent acidification activity, which may be responsible for a weaker LysoTracker staining in L286V AD cells in contrast to WT cells.

## DISCUSSION

The primary finding of the current study is that propofol, when administered above commonly used clinical concentrations ( $>50 \mu\text{M}$ ), sensitized AD neurons to impaired autophagy flux through decreased transport of the vATPase from the ER into the lysosome, after excessive  $\text{Ca}^{2+}$  release from the ER due to over activation of  $\text{InsP}_3\text{R}$  and/or RYR. Antagonists of  $\text{InsP}_3\text{R}$  (Xc) or RYR (dantrolene) alone ameliorated, while the combined use of Xc and dantrolene paradoxically worsened propofol toxicity in AD cells. Further impairment of autophagy flux by Bafilomycin in AD cells renders these neurons more vulnerable to propofol toxicity than WT cells, consistent to previous study in human stem cells [39]. This study illustrated the molecular mechanisms of propofol mediated neurotoxicity, and developed strategies to mitigate such harmful effects of general anesthetics in neurons featured with AD.

Adequate  $\text{Ca}^{2+}$  release from ER via  $\text{InsP}_3\text{Rs}$  and/or RYRs, and the subsequent elevation of  $[\text{Ca}^{2+}]_c$  plays a universal role on many cell functions, including, but not limited to ATP production, cell survival, cell cycles, neurogenesis, autophagy, and cognitive function [40]. However, abnormal and excessive  $\text{Ca}^{2+}$  release from the ER via  $\text{InsP}_3\text{Rs}$ /RYRs may result in impaired autophagy, mitochondria damage, cell arrest and death [41], which may be the mechanism of some pathology in neurodegenerative diseases [42]. It has been demonstrated that both  $\text{InsP}_3\text{Rs}$  and RYRs are excessively activated in AD due to abnormally increased receptor numbers [43], and the elevated receptor hypersensitivity to their agonists [44]. Abnormal and excessive  $\text{Ca}^{2+}$  release from the ER not only dangerously increases  $[\text{Ca}^{2+}]_c$  but also detrimentally decreases ER calcium contents. The former can overload mitochondria with  $\text{Ca}^{2+}$  and collapse membrane potential, leading to type 1 cell death, apoptosis. The latter may impair protein synthesis and secretion from the ER, causing cell damage. One of the impaired protein synthesis is that of the vATPase, which plays an important role in the uptake of  $\text{H}^+$  into the lysosome lumen and maintaining its acidity for normal lysosome function. Lack of efficient vATPase on the lysosome membrane in AD cells and resulting low  $\text{H}^+$ , further causes  $\text{Ca}^{2+}$  release from lysosome via TRPML1 calcium channel. This leads to increased  $[\text{Ca}^{2+}]_c$  and to a vicious cycle of the disruption of  $\text{Ca}^{2+}$  homeostasis in the ER, mitochondria and cytosolic space. These upstream pathologies can result in lysosome dysfunction and in the impairment of autophagy, which causes type 2 autophagy cell death or promotion of apoptosis (Fig. 5). Clearly, the  $\text{Ca}^{2+}$  release from the ER via  $\text{InsP}_3\text{Rs}$ /RYRs plays a dual role of both promoting cell survival and inducing cell damage, depending on the amount of  $\text{Ca}^{2+}$  released from the ER.

We have demonstrated in previous studies [45] that isoflurane at clinically relevant concentrations induced more significantly cell damage in L286V AD cells than in WT. Although previous studies demonstrated that inhalational anesthetics affect autophagy and cell survival via activation  $\text{InsP}_3\text{Rs}$ /RYRs [46], the molecular mechanisms that GAs effects on autophagy and cell survival is not clear. Our study here suggests that abnormal  $\text{Ca}^{2+}$  release from the ER, induced by excessive propofol, results in ER stress, decreased



vATPase, and impaired lysosome and autophagy function. Although propofol significantly decreased lysosome vATPase, it increased the overall intracellular acidity. We presumed that this increased acidity was not originated from lysosome and may be caused by significantly increased vATPase in early endosome (Fig. 3). It is well known that  $\text{Ca}^{2+}$  plays an important role in regulating fusion of multiple types of vesicle including endosome-lysosome fusion. It has been shown that high cytosolic calcium concentration suppressed vesicle fusion, and calcium channel blockers can attenuate saturated fatty acids induced autophagy arrest in mice by promoting autophagosome-lysosome fusion [47]. Therefore, either abnormally high cytosolic  $[\text{Ca}^{2+}]$  and/or low ER calcium level may contribute to defective lysosomal acidification and autophagosome-lysosome fusion, which may be responsible for lysosomal proteolytic failure and contribute to AD-related accumulation of autophagy and endosomal substrates and neurodegeneration. This defect of lysosomal function contribute to inefficient protein/peptide clearance, amyloid and tau pathology in AD [48].

Consistent with our previous study [46], the detrimental effects of propofol could be inhibited by antagonists for either  $\text{InsP}_3\text{Rs}$  or  $\text{RyRs}$  in AD cells. Unexpectedly, the combined use of antagonists for  $\text{InsP}_3\text{Rs}$  and  $\text{RyRs}$  not only provided no neuroprotection but also worsened  $\text{Ca}^{2+}$  dysregulation and promoted cell death in AD cells. It seems that simultaneous inhibition of both  $\text{InsP}_3\text{R/RyR}$  will impair its normal physiologic function and are detrimental to cells. Our results suggest that strategy for treatment of anesthesia toxicity in AD depends on adequate amelioration of  $\text{InsP}_3\text{R}$  or  $\text{RyR}$ . However, excessive inhibition of both receptors disrupts processes that regulate extracellular  $\text{Ca}^{2+}$  influx and normal  $\text{Ca}^{2+}$  release from the ER, and physiological function can be impaired.

It seems that the existing reduction of vATPase, and impaired lysosome and autophagy function in the AD cells, make them vulnerable to further decrease of vATPase induced by propofol and promotes cell death. Previous studies suggest that inhalational anesthetics, especially isoflurane, at clinically relevant concentrations, can induce significant  $\text{Ca}^{2+}$  release from ER via  $\text{InsP}_3\text{Rs}$  or  $\text{RyRs}$  [49] and cell damage. Propofol at clinically relevant concentrations rarely changes cytosolic  $\text{Ca}^{2+}$  concentrations and causes cell death, suggesting a less likely possibility of propofol disrupting intracellular  $\text{Ca}^{2+}$  homeostasis and a higher chance of cell survival than in inhalational anesthetics. The results from this study seems to support this notion since propofol, only at extremely high concentrations, much higher than clinically relevant concentrations, could impair lysosome acidity and function, damage autophagy function, and induce cell death. More studies are needed to investigate whether this data that a lower potency of propofol causes less cell damage than commonly used inhalational anesthetics is applicable to human beings. Particularly in human cells vulnerable to anesthesia toxicity, such as in AD and developing brains.

The scientific implication of this study is that maintenance of normal intracellular calcium homeostasis is important for cell survival, which was disrupted in AD cells by excessive calcium release from ER via over activation of  $\text{RyR/InsP}_3\text{R}$  due to presenlin-1 mutation. Approaches to ameliorate calcium dysregulation by inhibition of  $\text{RyR/InsP}_3\text{R}$  should be adequate, with excessive inhibition by simultaneous inhibition of both  $\text{RyR}$  and  $\text{InsP}_3\text{R}$  leading to pathological low cytosolic calcium concentrations unable to maintain

physiological need as a second messenger. Notably, the detrimental effects of propofol were observed only at high doses and/or the presence of bafilomycin. This may be relevant in elderly individuals due to declining autophagy function with aging. Nevertheless, taken together with previous studies [21, 50], the clinical implication of this study is that general anesthetics may cause neuronal damage more in AD than WT cells, with inhalational anesthetics more potent than propofol.

The limitations of this study include: 1) We did not measure the direct effects of propofol on autophagy function in the current study, as this has been investigated in human stem cells in previous study [39] and is not our current focus; 2) Lack of direct comparison between propofol and some commonly used inhalational anesthetics (e.g., sevoflurane, desflurane, isoflurane), which warrant further studies in the future; 3) Lack of further studies on molecular mechanisms of  $\text{Ca}^{2+}$  influx induced by the combined use of antagonists on  $\text{InsP}_3\text{R/RYR}$ . We plan to investigate this mechanism in detail in future studies; 4) The clinical relevance of this study is limited by the use of immortalized cell lines, which are not true neurons from CNS. Future studies may focus on the primary neuronal cultures or neurons directly from AD patients or controls.

In summary, propofol, at pharmacological concentrations caused cell damage in AD cells, sensitized by deficits in autophagy flux, via over activation of  $\text{InsP}_3\text{Rs}$  and/or  $\text{RYRs}$ . These pathological changes can be inhibited by antagonists of either receptors, but not the combination of both.

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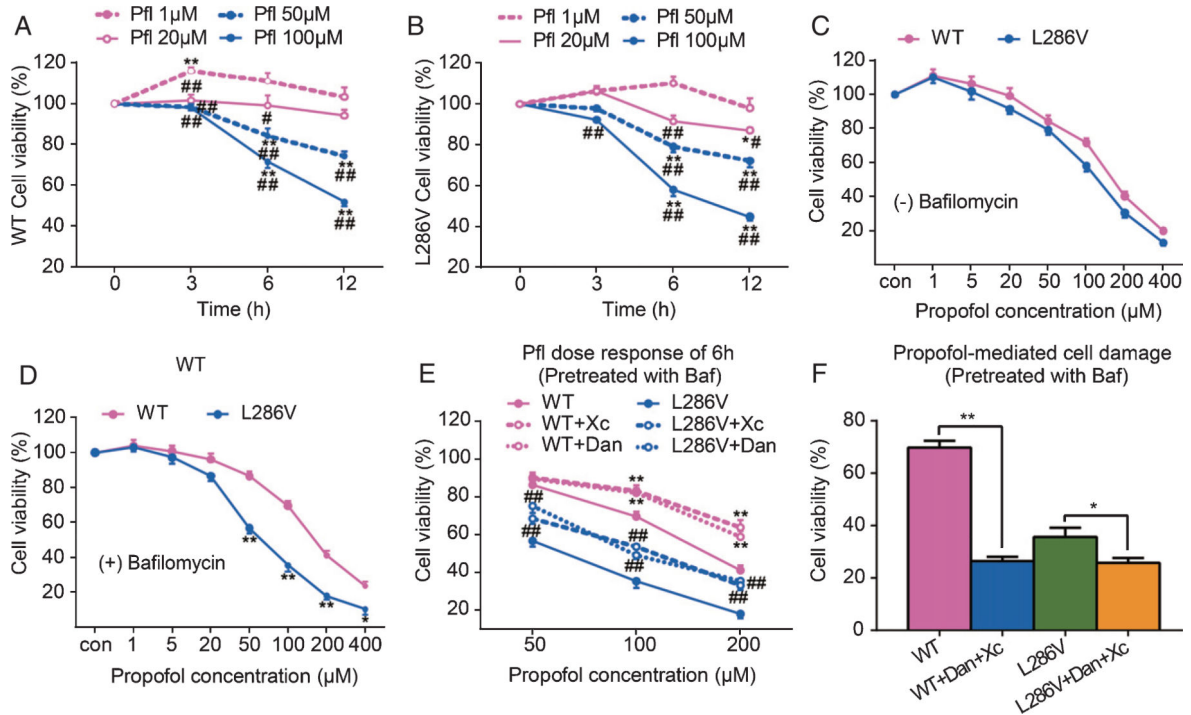


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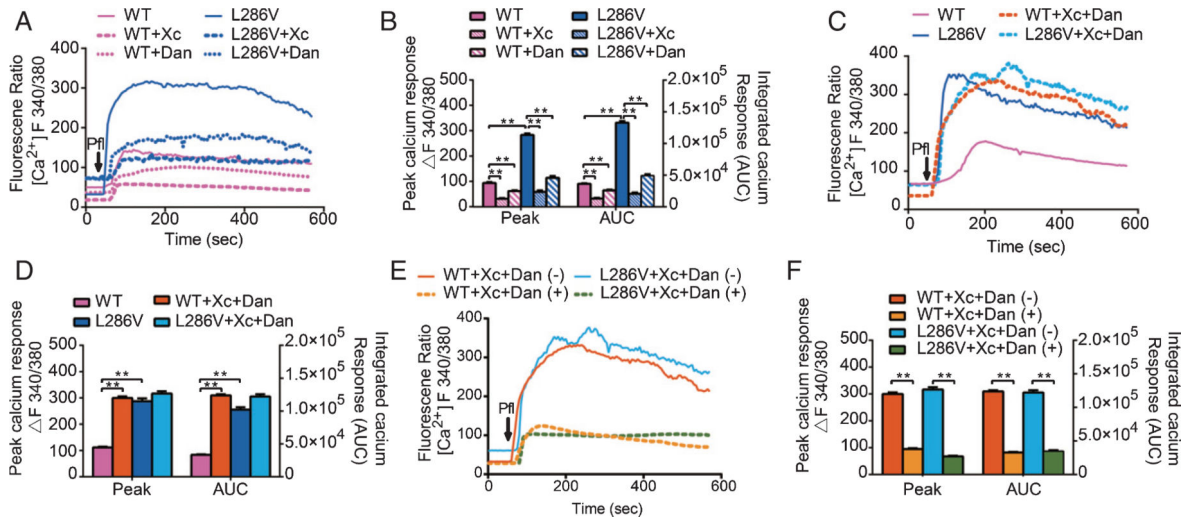
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**Fig. 1.**

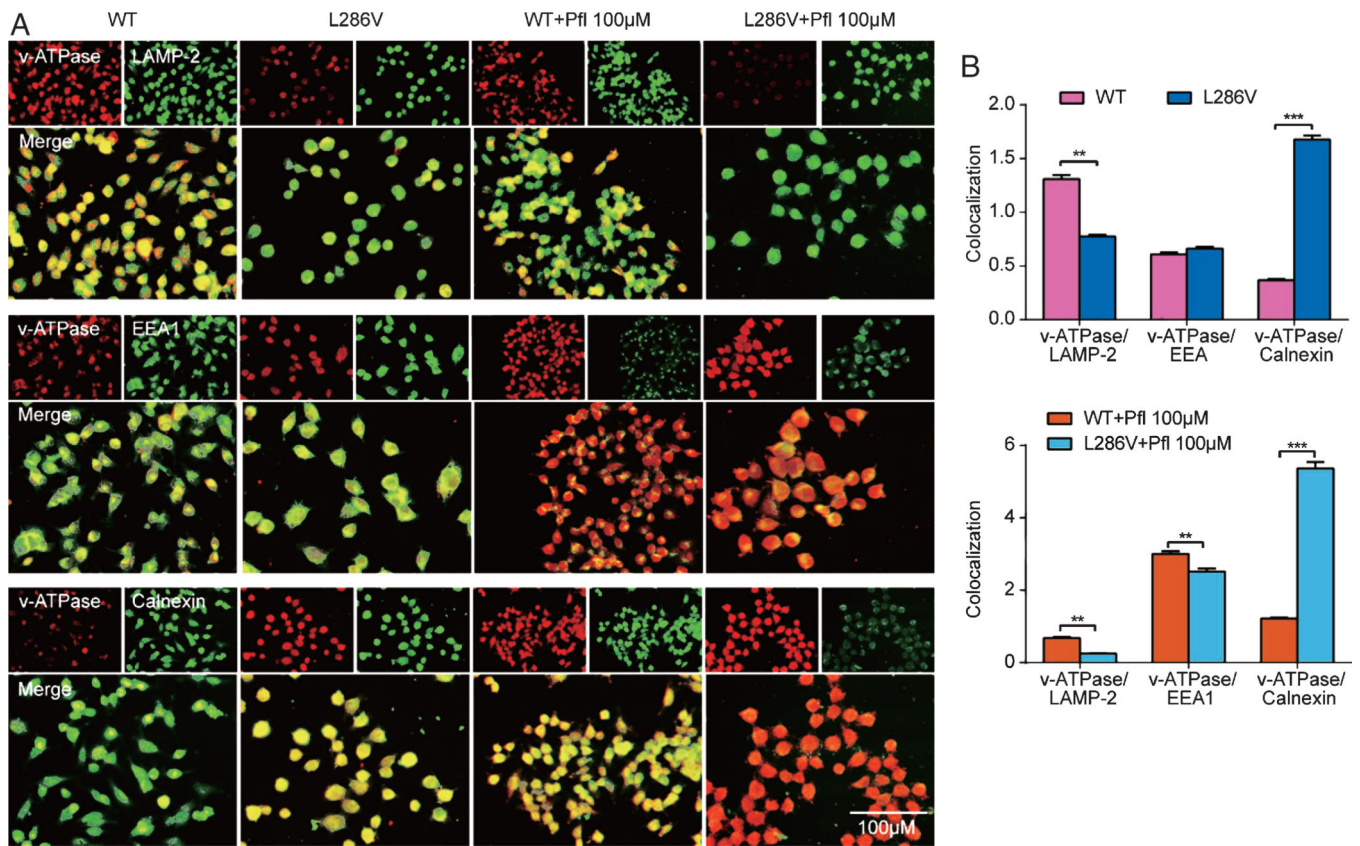
Propofol affects cell viability in an autophagy activity-dependent manner via activation of InsP<sub>3</sub> (InsP<sub>3</sub>R) or ryanodine (RyR) receptors. PC12 rat pheochromocytoma cells transfected with knocked in point mutation of presenilin 1 (L286V) or wild type (WT) controls were exposed to different concentrations of propofol (1 to 400 μM) for 3, 6, or 12 h. The Cell Counting Kit-8 (CCK-8) was used to determine cell viability. Propofol dose- and time-dependently affect cell viability in both types of cells equally, with low dose (1 μM) for up to 6 h tended to increase cell viability but high concentrations (over 50 μM) significantly reduced cell viability (A and B). \**p* < 0.01 and \*\**p* < 0.001 compared to the beginning time point at each propofol dose group (N = 3). #*p* < 0.01 and ##*p* < 0.001 compared to propofol (1 μM) group at each time point (N = 3). Impairment of autophagy flux by bafilomycin did not worsen the 6 h propofol mediated cell damage in WT cells (C), but significantly increased vulnerability of L286V cells to propofol dose-dependent toxicity (D). \**p* < 0.01 and \*\**p* < 0.001 compared to WT (N = 3). The propofol toxicity in autophagy-disrupted L286V cells was inhibited by antagonists for InsP<sub>3</sub>Rs (E, xestospongins C, Xc 1 μM) or RyRs (E, dantrolene, 30 μM), but paradoxically worsened by the combined use of Xc and dantrolene (F). \**p* < 0.05 and \*\**p* < 0.01 (N = 3). All data in the figure are expressed as the mean ± SEM from at least three separate experiments with triplicates and analyzed by two-way ANOVA followed by Tukey post multiple comparison tests.



**Fig. 2.**

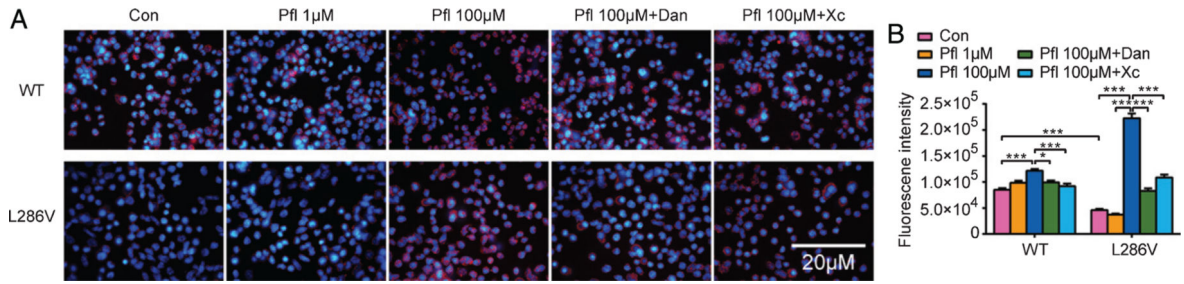
Propofol increased cytosolic  $Ca^{2+}$  concentrations ( $[Ca^{2+}]_i$ ) more in L286V than WT cells via activation of  $InsP_3$  ( $InsP_3R$ ) or ryanodine (R<sub>YR</sub>) receptors. A) Average  $[Ca^{2+}]_i$  response to 200  $\mu$ M propofol (arrow) in WT or L286V cells in the presence or absence of antagonists for  $InsP_3R$ s (Xestospongin C, Xc 1  $\mu$ M) or R<sub>YR</sub>s (dantrolene or Dan, 30  $\mu$ M). B) Propofol increased peak  $[Ca^{2+}]_i$  and integrated  $Ca^{2+}$  calcium response represented by area under the curve (AUC) significantly more in L286V than in WT cells, which were significantly inhibited by Xc or dantrolene alone. C) Average  $[Ca^{2+}]_i$  response to 200  $\mu$ M propofol (arrow) in WT or L286V cells in the presence or absence of combined use of Xestospongin C and dantrolene (Xc+Dan). D) Combined use of Xc and Dan paradoxically elevated both the peak and AUC of  $[Ca^{2+}]_i$ , only in WT but not L286V cells. E) Average  $[Ca^{2+}]_i$  response to 200  $\mu$ M propofol (arrow) with pretreatment of both xestospongin C and dantrolene (Xc+Dan) in WT or L286V cells in the presence or absence of EGTA, a calcium chelator to remove extracellular  $Ca^{2+}$  in the measurement buffer. F) Removal of extracellular  $Ca^{2+}$  from the buffer nearly abolished the increased  $[Ca^{2+}]_i$  induced by the propofol in the presence of combined inhibition of both  $InsP_3R$  and R<sub>YR</sub>. All data (B, D, F) are expressed as the mean  $\pm$  SEM from two or three separate experiments of at least twenty individual cells (*N* = 3) and analyzed by one-way ANOVA followed by Tukey multiple comparison tests. \**p* < 0.01 and \*\**p* < 0.001.



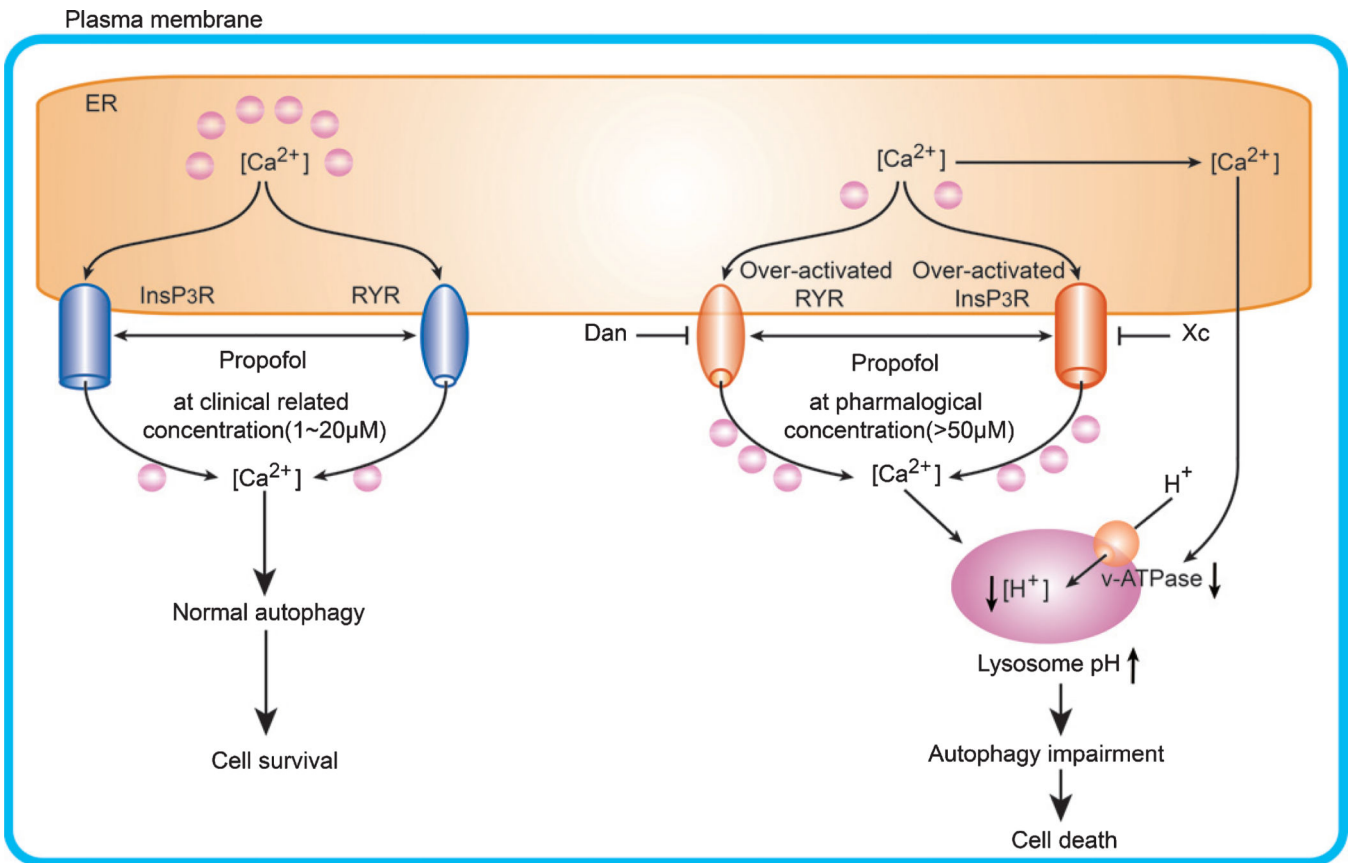


**Fig. 3.** Propofol decreased lysosome vATPase in L286V Alzheimer’s disease cells by over activation of  $InsP_3$  ( $InsP_3R$ ) or ryanodine (RZR) receptors. **A)** Double-immunofluorescence labeling shows the colocalization of vATPase (V0a1 subunit) and lysosome (LAMP-2), or early endosome (EEA1), or ER (calnexin) in WT or L286V AD cells. Scale bar = 50  $\mu m$ . **B)** Compared to WT controls, vATPase V0a1 subunit and lysosomal marker, LAMP-2 show little colocalization in L286V AD cells. Compared to WT controls, propofol at high concentration (Pfl 100  $\mu M$ ) significantly minimally colocalized vATPase V0a1 and lysosome marker, LAMP-2, but increased vATPase V0a1 in ER and early endosome. All data are expressed as the mean  $\pm$  SEM from at least three separate experiments (N = 3) of at least twenty individual cells and analyzed by unpaired *t* test followed by Tukey multiple comparison tests. \**p* < 0.01 and \*\**p* < 0.001.





**Fig. 4.** Propofol significantly increased acidity of lysosome in L286V cells by over activation of InsP<sub>3</sub> (InsP<sub>3</sub>R) or ryanodine (RZR) receptors. A) Representative pictures of lysosome acidity (red staining) determined by LysoTracker. With or without propofol treatment (1 versus 100 µM) and in the presence or absence of antagonists for InsP<sub>3</sub>Rs (xestospongin C, Xc) or RZRs (dantrolene, Dan). Scale bar = 20 µm. B) Propofol only at non-clinical high concentrations (100 µM) increased acidity, more significantly in L286V cells, which were abolished by Xc or Dan. All data are expressed as the mean ± SEM from at least three separate experiments (N = 3) of at least twenty individual cells and analyzed by two-way ANOVA followed by Tukey multiple comparison tests. \**p* < 0.01 and \*\**p* < 0.001.



**Fig. 5.** Propofol affect cell survival in a dose- and lysosome  $H^+$  homeostasis dependent manner via activation of InsP<sub>3</sub>Rs and/or RYRs. Propofol at clinically relevant concentrations (1–20  $\mu M$ , left side) adequately activate InsP<sub>3</sub>R and/or RYR, supporting normal lysosome  $H^+/Ca^{2+}$  homeostasis and cell survival. However, excessive activation of InsP<sub>3</sub>Rs and/or RYRs by propofol at pharmacological concentrations (>50  $\mu M$ , right side) and associated depletion of ER  $Ca^{2+}$  together with abnormal elevation of cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_c$ ) resulted in the disruption of lysosome  $H^+/Ca^{2+}$  homeostasis, and impairment of endosome-lysosome fusion and autophagic dysfunction, eventually leading to cell death.