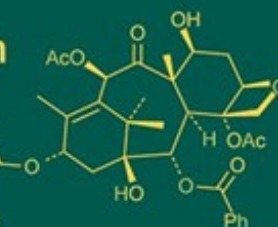
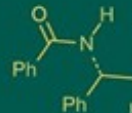


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Scopolamine-induced cholinergic dysfunction perturbs lysosomal homeostasis in the prefrontal cortex of mouse brain

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ABSTRACT

Scopolamine-induced cholinergic dysfunction is widely used to model cognitive impairment; however, its impact on lysosomal homeostasis remains poorly understood. Given the prominent role of the prefrontal cortex in cognitive function and its substantial cholinergic innervation, this region provides a relevant context for investigating cellular responses to cholinergic disruption. The present study investigated early alterations in the acidic vesicular compartment and lysosomal homeostasis following scopolamine exposure in LN229 glial cells and the mouse prefrontal cortex. Scopolamine (10 μ M) induced a time-dependent reduction in LysoTracker fluorescence, with an early decline at 1 h and a significant reduction at 2 h, indicating rapid perturbation of the acidic vesicular compartment. In vivo, scopolamine-challenged mice (1 mg/kg) exhibited altered neuronal morphology and disrupted cortical architecture in the prefrontal cortex. Western blot analysis revealed reduced expression of LAMP1, Cathepsin B and Cathepsin D, accompanied by increased TFEB expression, suggesting impaired lysosomal function with a compensatory transcriptional response. Collectively, these findings identify lysosomal dysregulation as an early and previously underexplored cellular response to scopolamine-induced cholinergic disruption, potentially contributing to cellular vulnerability in the prefrontal cortex.

Keywords: Scopolamine, Cognitive dysfunction, LysoTracker, LAMP 1, TFEB, Cathepsin, Prefrontal Cortex

1. Introduction

Cholinergic neurotransmission is the process by which neurons communicate using the neurotransmitter acetylcholine. This process is important for attention, learning, memory, and executive functions (Luchicchi *et al.*, 2014) [1]. Any disruption of central muscarinic signaling creates cognitive impairment (Szczurowska *et al.*, 2022) [2]. Scopolamine, a non-selective muscarinic acetylcholine receptor antagonist that can readily cross the blood-brain barrier, is widely employed by researchers as a pharmacological tool to induce cholinergic dysfunction and memory impairment (Klinkenberg *et al.*, 2010; Chen *et al.*, 2020) [3, 4]. The scopolamine model may not reproduce all the complete pathological spectrum of Alzheimer's disease or any other dementias but it can induce a reproducible cognitive phenotype for investigating cellular consequences of impaired cholinergic signaling (Klinkenberg *et al.*, 2010; Blokland, 2022) [3, 5]. Interestingly, the effects of scopolamine extend beyond transient muscarinic receptor blockade. Experimental studies have shown alterations in oxidative homeostasis, mitochondrial function, inflammatory signaling, apoptosis, and neuronal integrity following scopolamine exposure (Chen *et al.*, 2020; Wong-Guerra *et al.*, 2017; San Tang, 2019; Olayinka *et al.*, 2022) [4, 6, 7, 8].

The prefrontal cortex (PFC) is the anterior region of the cerebral cortex involved in higher-order cognitive functions such as attention, working memory, decision-making and executive control (Friedman *et al.*, 2022; Levy, 2024) [9, 10]. It receives substantial cholinergic projections from basal forebrain nuclei, particularly the nucleus basalis and related cholinergic cell groups, allowing acetylcholine to modulate cortical neuronal activity, attention and cognitive processing (Ballinger *et al.*, 2016; Ananth *et al.*, 2023) [11, 12]. Consequently, the PFC represents an important cortical region in which disruption of muscarinic cholinergic signaling by scopolamine can be investigated to understand the cellular mechanisms underlying cognitive dysfunction (Vijayaraghavan *et al.*, 2021; Cools *et al.*, 2022) [13, 14].

Glial cells are essential components of the central nervous system that support neuronal function, maintain tissue homeostasis, and regulate neural communication (Allen *et al.*, 2018) [15]. In the prefrontal cortex (PFC), astrocytes and microglia play critical roles in neurotransmitter regulation, metabolic support, synaptic remodeling, and immune surveillance. Proper glial-neuronal interactions are therefore essential for maintaining the neural circuits underlying attention, learning, memory, and executive functions. Dysregulation of

glial activity in the PFC can disrupt neuronal homeostasis and synaptic plasticity, potentially contributing to cognitive dysfunction (Zhao *et al.*, 2022; Ren *et al.*, 2025) [16, 17].

Lysosomes are dynamic intracellular organelles that integrate macromolecular degradation, autophagic clearance, membrane trafficking, nutrient sensing, and cellular stress responses (Ballabio *et al.*, 2020; Settembre *et al.*, 2024; Radulovic *et al.*, 2026) [18, 19, 20]. Maintenance of an acidic lysosomal lumen is critical for the activity of lysosomal hydrolases and efficient degradation of intracellular cargo (Canton *et al.*, 2015; Kim *et al.*, 2025; Zhang *et al.*, 2025) [21, 22, 23]. This function is especially important in long-lived post-mitotic neurons and surrounding glial cells that depend on efficient lysosomal clearance for maintenance of protein and organelle quality (Damme *et al.*, 2015; Kreher *et al.*, 2021; Fleming *et al.*, 2022) [24, 25, 26]. Any disturbance in lysosomal homeostasis is an important contributor to neuronal vulnerability and neurodegeneration (Kim *et al.*, 2025; Drobny *et al.*, 2022; Nixon *et al.*, 2024; Nixon, 2024) [22, 27, 28, 29].

In the lysosome the Lysosome-associated membrane protein 1 (LAMP1) is a major component of the lysosomal membrane and is closely associated with maintenance of the lysosomal compartment (Eskelinen, 2006; Saftig *et al.*, 2009; Bhattacharya *et al.*, 2023) [30, 31, 32]. The lysosomal proteases such as Cathepsin B (Cat B) and Cathepsin D (Cat D) participate in intracellular protein turnover and neuronal proteostasis (Drobny *et al.*, 2022; Vidoni *et al.*, 2016; Marques *et al.*, 2020; Lewandowski *et al.*, 2025) [27, 33, 34, 35]. Lysosomal demand is coordinated at the transcriptional level by transcription factor EB (TFEB), the principal regulator of the coordinated lysosomal expression and regulation (CLEAR) network that regulates lysosomal biogenesis, autophagy, and cellular clearance pathways. It responds dynamically when lysosomal degradative capacity is challenged (Sardiello *et al.*, 2009; Palmieri *et al.*, 2011; Settembre *et al.*, 2011; Sardiello, 2016; Shariq *et al.*, 2024) [36, 37, 38, 39, 40]. So any alteration of lysosomal acidity, membrane-associated proteins, lysosomal proteases, or TFEB expression could provide an important mechanistic link between scopolamine-induced cellular stress and progressive impairment of intracellular proteostasis.

Scopolamine-induced neuronal degeneration and cellular injury are demonstrated within the prefrontal cortex (Wong-Guerra *et al.*, 2017; Olayinka *et al.*, 2022; Memudu *et al.*, 2022; Park *et al.*, 2024) [6, 8, 41, 42]. However, little is known about whether scopolamine can directly influence the endolysosomal system and lysosomal proteostasis.

Based on these available literature, we hypothesized that scopolamine-induced cholinergic stress produces an early disturbance of the acidic vesicular compartment and subsequently evokes alterations in lysosomal machinery in the prefrontal cortex, involving compensatory components of the lysosomal response. So to test this hypothesis, we first adopted an *in vitro* approach by employing scopolamine challenged human glioblastoma-derived LN-229 cells and LysoTracker to understand indication of alterations in acidic vesicular homeostasis. Second, we used an *in vivo* approach of scopolamine induced mouse model to understand the changes in the prefrontal cortex and important molecular components of different levels of lysosomal organization like lysosomal membrane protein LAMP1, the proteolytic enzymes Cat B and Cat D, and the lysosomal transcriptional regulator TFEB by histology and Western blotting. By integrating these methods, the study aimed to determine whether lysosomal dysregulation constitutes an additional cellular component of scopolamine-induced pre-frontal cortical stress.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in the present study were procured from the respective manufacturers and are listed below with their catalogue numbers. Chemicals obtained from Sigma-Aldrich (USA) included Alanine-Glutamine (G8541), Bradford reagent (B6916), Paraformaldehyde (16005), Protease/Phosphatase inhibitor (PPC1010), Triton X-100 (93443), and Tween 20 (P9416). Reagents

obtained from Thermo Fisher Scientific (USA) included LysoTracker™ Deep Red dye (L12492), Pierce™ 5X sample buffer/lane marker non-reducing sample buffer (39001), Pierce™ BCA Protein Assay Kit (23227), Qubit™ Protein Broad Range Assay Kit (A50668), Restore™ Western Blot Stripping Buffer (21059), SuperSignal™ West Femto Maximum Sensitivity Substrate (34094), and DNeasy PowerWater Pro Kit (14900-100-NF). Gibco (NY, USA) supplied Dulbecco's Modified Eagle Medium (DMEM; 12800017) and fetal bovine serum (FBS; 10270-106). Calbiochem, Merck (Germany) supplied Scopolamine Hydrobromide (CAS No. 114-49-8), while HiMedia (India) supplied Penicillin-Streptomycin (A001) and Mayer's Hematoxylin (S058). EMD-Millipore (MA, USA) supplied Radioimmunoprecipitation Assay (RIPA) buffer (20-188), and Bio-Rad Laboratories (California, USA) supplied Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (1610375). Histological and processing reagents obtained from Spectrum (India) included Eosin Yellow (E0106), Paraffin Wax 58–60 °C (P0105), D.P.X. Mountant (D0101), Glycerol (G0105), Glacial Acetic Acid (A0101), and Xylene (Sulphur Free; X0104). Rankem Chemicals (Haryana, India) supplied Ammonia Solution (A0280) and Formaldehyde Solution 37% (F0090). SRL (Maharashtra, India) supplied Isopropanol (67800), whereas Spectrochem (India) supplied Methanol (CAS No. 67-56-1). Antibodies used in the study is listed in table 1.

2.2. Cell culture

LN229 cell line, a human glioblastoma multiforme (GBM) line derived from an aggressive brain tumor were purchased from the National Centre for Cell Science (NCCS), Pune. The LN229 cells were cultured using Dulbecco's modified Eagles medium (DMEM) with 10% Fetal bovine serum (FBS), 1% antibiotic and 2 mM Alanine-Glutamine and maintained at 37 °C in the presence of 5% CO₂ and 95% humidity.

For experiment, cell culture dishes at 85-90% confluency shifted to low serum conditions (0.5% FBS) and incubated for 30 minutes at 37 °C. The cells were then challenged with scopolamine (10 µM) and incubated for 1 hour or 2 hours. Following all treatments, lysosomal staining was done by LysoTracker (50 nM). Imaging was performed using an EVOS FLoid™ Cell imaging station (Invitrogen, Thermo Fisher Scientific, USA), and analysed using ImageJ 1.53 software (NIH).

2.3. Animals

10-12 week old healthy female Swiss albino mice weighing 25-28g body weight were used for the study. Mice acclimated for one week before the experiments were housed under standard conditions. They were given a standard pellet diet and water *ad libitum*. All the experimental protocols were followed as per the committee's guidelines for the purpose of controlling and supervising experiments on animals (CPCSEA), Government of India. The experimental protocol for animals was approved by the Institutional Animal Ethical Committee (IAEC) of the University of Kerala, Thiruvananthapuram, Kerala (Approval number IAEC 1-KU-21/2021-BCH-VV (Szczurowska *et al.*, 2022) [2]; dated 12-03-2021).

2.3.1. Experimental design

Animals were randomly divided into two groups, with six animals in each group ($n = 6$). Group I (Normal control) received 0.9% normal saline intraperitoneally (i.p.), whereas Group II (Scopolamine; SCO) received scopolamine at a dose of 1 mg/kg body weight via the i.p. route. The experimental period was 7 days, during which the respective treatments were administered according to the experimental protocol. On the 7th day, a single dose of scopolamine (1 mg/kg, i.p.) was administered to the SCO group. At the end of the study, mice were anaesthetized using ketamine (80 mg/kg) and Xylazine (20 mg/kg) combination and perfused with 10% formalin. Then, the brains were collected from each group preserved in 10% formalin for histopathology analysis. For western blot analysis brains were perfused with normal saline and kept in -80 degree deep freezer.

2.4. Histological analysis of prefrontal cortex

After harvested, brain tissues were gently washed with saline and fixed in 10% formalin for 12–24 h. Following fixation, the tissues were washed under running water for 5 min and dehydrated through a graded series of isopropyl alcohol (50%, 70%, 80%, and 100%; 1 h each). The dehydrated tissues were then cleared in xylene for 2 hours to remove alcohol and facilitate wax penetration. The tissues were embedded in paraffin blocks, which were allowed to cool before sectioning. Sections of 5 µm thickness were prepared using a rotary microtome (Weswox, SMT-1090A), the sections were floated on a warm water bath, and placed on albuminized glass slides. Any excess water was removed using filter paper. The slides were then placed in an oven at 60 °C for 1 hour to ensure proper adhesion of the sections to the slides.

For hematoxylin and eosin (H&E) staining, sections were deparaffinized in three changes of xylene (15 min each) and rehydrated through descending concentrations of alcohol. The sections were stained with Mayer's hematoxylin for 5–10 min, washed in running tap water for 15 min, and counterstained with eosin for 1 min. The stained sections were dehydrated through graded alcohol, cleared in xylene, and mounted with DPX mounting medium. Coverslips were applied, and the sections were examined under a transmitted-light polarising microscope (TLMP-5, CX31).

2.5. Immunoblotting

After the isolation of prefrontal cortex, tissue (40 mg) was homogenized in ice-cold RIPA lysis buffer with protease and phosphatase inhibitors (1% v/v) followed by sonication at 50% amplitude for 10 sec on and 20 sec off (Sonics & Materials Inc., vcx130, USA). The homogenate was incubated on ice for 30 minutes with gentle agitation every 10 minutes and was centrifuged at $14,000 \times g$ for 15 minutes at 4 °C. The supernatant was carefully collected and transferred to new pre-chilled tubes.

Protein Estimation using Qubit™ Protein estimation using Qubit™ was performed according to the manufacturer's recommendations.

Briefly, standards were prepared by diluting 20 µl of the supplied Qubit™ Protein BR Assay Reagent into 150 µl of Qubit™ Protein BR Assay Buffer. Protein samples and blanks were also prepared by adding 20 µl of the sample to 150 µl of Qubit™ Protein BR Assay Buffer. This was followed by the addition of 30 µl of Qubit™ Protein BR Assay Reagent. The samples were vortexed immediately and incubated at room temperature for 30 minutes. The samples and standards were then read using a Qubit™ 4 Fluorometer (Thermo Fischer Scientific, USA) with the Protein Broad Range settings.

Protein estimation was performed to ensure equalization, followed by the samples (40 µg protein) were boiled at 90 °C for 10 minutes in 5X Pierce™ lane marker non-reducing sample buffer to denature the proteins. These were loaded onto the wells of a 10% SDS-PAGE gel. A prestained protein standard was included in one lane. An electrophoretic run was conducted at 80 V for 30 minutes, and increased to 120 V upon transition from the stacking to the resolving gel until the dye front reached the bottom of the gel.

After electrophoresis, the resolving gel was transferred onto nitrocellulose membranes (0.45 µm) using a blotting apparatus (Bio-Rad) at 100 V for 2 hours at 4 °C in 1X transfer buffer. The membrane was then removed, washed and blocked overnight with 5% BSA in Tris-buffered saline (pH 7.4, TBS). Membranes were incubated overnight at 4 °C with primary antibody were listed in table 2.2. Membranes were washed thrice with TBS containing 0.1% Tween-20 (TBST) and incubated with HRP-conjugated secondary antibody incubation for 2 hours at room temperature. Chemiluminescent detection was performed using a Bio-Rad ChemiDoc XRS+ apparatus after the addition of Super Signal ITM West Femto Maximum Sensitivity Substrate. Membranes were stripped for reprobing using Restore™ Western Blot Stripping Buffer, reblocked, reprobed with antibody and developed. The blots were analysed using ImageJ 1.53 software (NIH), and graphs were plotted using GraphPad Prism 5.0 software, with beta-actin as the normalisation control.

Table 1. Antibodies used for the Study

Sl.No.	Antibody	Dilution	Incubation
LAMP1 #3243 Cell Signaling Technologies, Danvers, Massachusetts, USA		1:1300	4 °C
Cat D #88239 Cell Signaling Technologies, Danvers, Massachusetts, USA		1:1300	4 °C
Cat B #31718 Cell Signaling Technologies, Danvers, Massachusetts, USA		1:1300	4 °C
TFEB #83010 Cell Signaling Technologies, Danvers, Massachusetts, USA		1:1300	4 °C
Beta Actin #4970 Cell Signaling Technologies, Danvers, Massachusetts, USA		1:1300	4 °C
Anti-Rabbit IgG , HRP-Linked Antibody (secondary Antibody) #7074 Cell Signaling Technologies, Danvers, Massachusetts, USA		1:4000	Room Temperature

2.6. Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Analyses were conducted using Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA). Statistical analysis was per-

formed using *t* test to identify significant differences among groups. The *p*-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Scopolamine reduces LysoTracker fluorescence in LN-229 cells

To determine whether scopolamine alters the acidic intracellular vesicular compartment, LN-229 cells were exposed to scopolamine and acidic vesicles were assessed using LysoTracker fluorescence microscopy. Representative fluorescence images and corresponding quantitative analyses are shown in Fig. 1(A-D). Compared with untreated control cells, scopolamine-challenged cells showed a significant reduction in LysoTracker fluorescence as early as 1 h after treatment (Fig. 1A''-B'', 1E, $p < 0.0001$). Following 2 h of scopolamine exposure, a more pronounced reduction in LysoTracker fluorescence was observed (Fig. 1C''-D''). Quantitative analysis demonstrated a statistically significant decrease in fluorescence compared with untreated cells (Fig. 1F, $p < 0.0001$). Thus, scopolamine exposure produced a time-dependent reduction in the LysoTracker-positive acidic vesicular signal in LN-229 cells, with significant changes as early as 1 h.

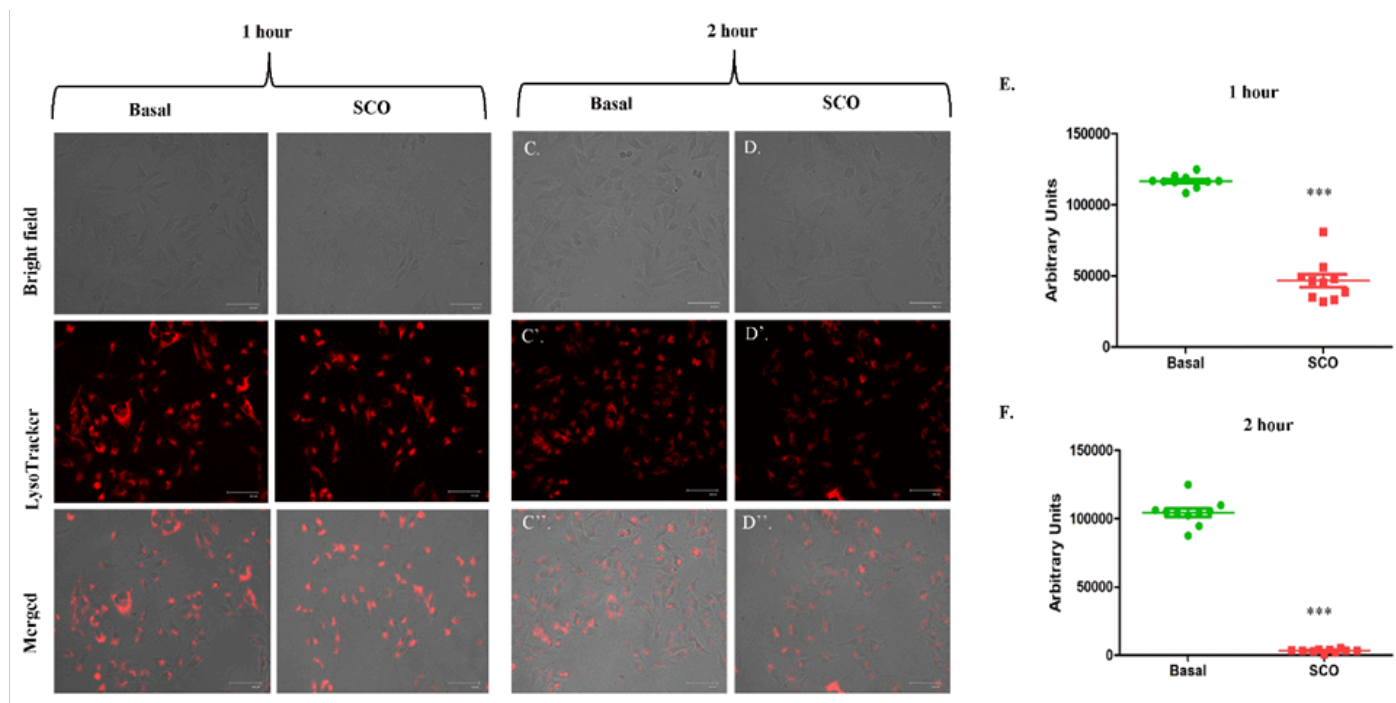


Figure 1. Scopolamine reduces LysoTracker fluorescence in LN-229 cells. (A-D) Representative images of bright field image of LN229 cells, (A'-D') Representative LysoTracker deep red image, (A''-D'') LysoTracker deep red merged image of LN229 cells merged with brightfield at 20X magnification, (E-F) Analysis of LysoTracker fluorescence intensity expressed as arbitrary units by Image J (NIH) at First Hour and Second hour respectively ($p < 0.0001$, $n = 30$).

3.2. Scopolamine induces histopathological alterations in the mouse prefrontal cortex

H&E staining of the prefrontal cortex sections of mouse brains revealed distinct morphological differences between control and scopolamine-challenged mice (Fig. 2). In the control group, the prefrontal cortex exhibited well-preserved cortical architecture with a relatively uniform distribution of neuronal profiles and an intact neuropil (Fig. 2A-A'). Neuronal cells displayed generally preserved morphology with distinct nuclei and maintained tissue organization. No prominent neuronal degeneration, vacuolation, or marked disruption of cortical

organization was observed. In contrast, the scopolamine-challenged group exhibited marked histopathological alterations in the prefrontal cortex (Fig. 2B-B'). The cortical tissue showed neuronal profiles with increased neurodegeneration (yellow arrow), irregular distribution of neuronal profiles, and focal disruption of the normal neuropil architecture. Apparently intact neuronal profiles were comparatively reduced, and focal areas of vacuolation (red asterisk) were evident within the cortical parenchyma. Overall, scopolamine challenge was associated with prominent alterations in neuronal morphology, focal vacuolation, and disruption of normal prefrontal cortical architecture.

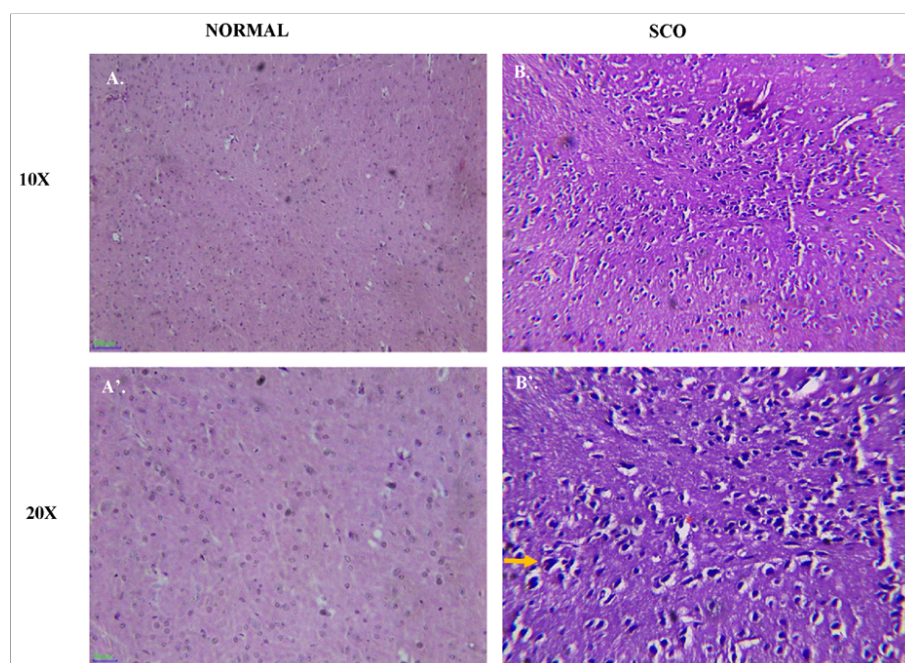


Figure 2. Scopolamine induces histopathological alterations in the mouse prefrontal cortex. Representative photomicrographs of Hematoxylin and Eosin (H&E) -stained sections of the mouse cerebral cortex of the different study groups. A-B: 20X magnification (Scale: 100 μ m) and zoomed A'-B': 40X magnification (Scale: 50 μ m). Neurodegeneration (yellow arrow); vacuolation (red asterisk).

3.3. Scopolamine decreases LAMP1 expression in the mouse prefrontal cortex

Western blot analysis was performed to assess the expression of lysosome-associated membrane protein 1 (LAMP1) in the prefrontal cortex of control and scopolamine-challenged mice. Normal showed presence of both glycosylated and non-glycosylated LAMP1 protein expression (Fig. 3A, 3a-b). Scopolamine challenge significantly reduced both glycosylated LAMP1 (Fig. 3a) and non-glycosylated LAMP1 (Fig. 3b) compared to control group ($p < 0.05$).

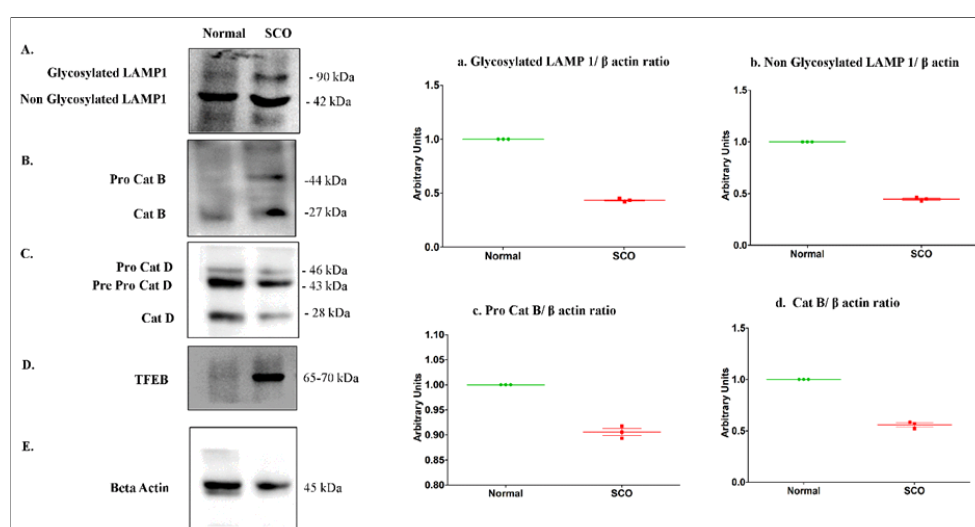
3.4. Cat B and Cat D exhibit impaired lysosomal enzyme activity following scopolamine challenge

Western blot analysis was performed to assess the expression of the lysosomal proteases Cat B and Cat D in the prefrontal cortex of control and scopolamine challenged mice. Normal showed presence of both Cat B (Pro Cat B and Cat B) and Cat D (Pro Cat D, Pre pro Cat

D and Cat D) protein expression (Fig. 3B, 3c-g). Scopolamine challenge significantly reduced both Pro Cat B (Fig. 3B, 3c) and Cat B (Fig. 3B, 3d) protein expression compared with control animals ($p < 0.05$). Similarly, Pro Cat D (Fig. 3C, 3e), Pre pro Cat D (Fig. 3C, 3f) and Cat D (Fig. 3C, 3g) protein expression was significantly decreased following scopolamine challenge compared with the control group ($p < 0.05$).

3.5. TFEB expression is increased in the prefrontal cortex following scopolamine challenge

Western blot analysis was performed to assess the expression of transcription factor EB (TFEB) in the prefrontal cortex control and scopolamine challenged mice. Normal control showed presence of TFEB protein expression (Fig. 3E, 3h). Scopolamine challenge significantly increased TFEB protein expression (Fig. 3E, 3h) in prefrontal cortex as compared with control animals ($p < 0.05$).



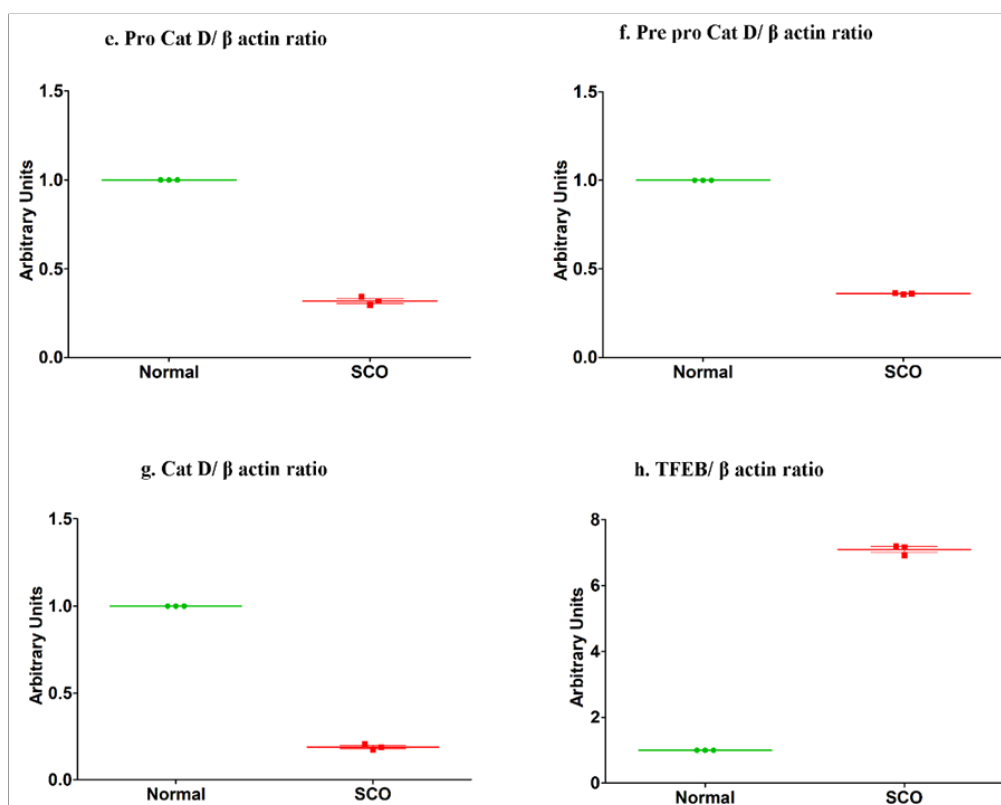


Figure 3. Effect of scopolamine on lysosomal protein expression in the prefrontal cortex. Representative immunoblots of LAMP1, Cat B, Cat D and TFEB. Densitometry analysis of immunoblot data showing relative protein expression normalized to β -actin across experimental groups. The band intensity (target protein/ β -actin) were calculated by Image J (NIH) software.

4. Discussion

The present study demonstrates that scopolamine challenge is associated with an early disturbance of the acidic vesicular compartment in vitro and with histopathological and lysosome-associated molecular alterations in the mouse prefrontal cortex. The major observations of the study are: (i) a rapid time-dependent reduction in LysoTracker fluorescence in scopolamine-treated LN-229 cells; (ii) prominent histopathological alterations in the prefrontal cortex following scopolamine challenge; (iii) decreased LAMP1 expression; (iv) differential regulation of the lysosomal proteases Cat B and Cat D; and (v) increased TFEB protein expression. Together these findings indicate that scopolamine-associated cellular stress involves the lysosomal system and is characterized by dysregulated lysosomal homeostasis. One of the earliest changes observed in the in vitro study was the reduction in LysoTracker fluorescence in LN-229 glial cells. Glial cells have highly active endolysosomal systems and contribute critically to cellular clearance and maintenance of cellular homeostasis (Kreher *et al.*, 2021) [25]. LysoTracker fluorescence reduction was observed within 1 h of scopolamine challenge and became statistically significant by 2 h, indicating that alteration of the acidic vesicular compartment occurs soon after scopolamine exposure. LysoTracker dyes are acidotropic fluorescent probes that accumulate inside acidic intracellular compartments, particularly late endosomes and lysosomes. Their accumulation depends mainly on maintenance of the acidic luminal environment generated by the lysosomal proton gradient (Canton *et al.*, 2015) [21]. Therefore, reduced LysoTracker fluorescence can indicate altered lysosomal acidification, disruption of the proton gradient, changes in acidic indicate a perturbation of the acidic endolysosomal compartment that occur early to contribute to alterations in intracellular proteostasis. So, we conducted an in vivo study where mice were challenged with scopolamine (Yadang *et al.*, 2020) [43] and investigated the lysosomal changes in the prefrontal cortex of scopolamine-challenged mice. The prefrontal cortex of the brain is a region of heterogeneous population of glial cells, including astrocytes, microglia, oligodendrocytes and their precursors, whose abundance and distribution vary across different regions of the mouse brain (Tan *et al.*, 2020;

Ero *et al.*, 2018) [44, 45]. Here we observed alterations in LAMP1, Cat B, Cat D and TFEB observed in whole prefrontal cortical tissue and this may be responses arising from either glial populations or neuronal cells or even both. Histological alterations confirm that neuronal populations in the prefrontal cortex were also affected with scopolamine challenge. Scopolamine is a muscarinic acetylcholine receptor antagonist extensively used to induce cholinergic dysfunction and cognitive deficits in experimental models (Chen *et al.*, 2020) [4]. There are accumulating evidence which indicate that prolonged scopolamine challenge produces cellular consequences beyond transient receptor blockade like oxidative stress, mitochondrial dysfunction, inflammatory responses and neuronal injury (Olayinka *et al.*, 2022; Muhammad *et al.*, 2019; Kukla *et al.*, 2026) [8, 46, 47]. Olayinka *et al.* (2022) [8] demonstrated that scopolamine challenge caused neuronal loss and neurodegenerative alterations involving both the hippocampal regions and prefrontal cortex, together with increased neuroinflammatory mediators. Similarly, Memudu *et al.* (2022) [41] reported pyramidal neuronal degeneration and structural alterations in the prefrontal cortex of scopolamine-treated rats. These observations are consistent with the morphological abnormalities observed in the present study.

LAMP1 is one of the major glycoproteins of the lysosomal membrane and is extensively used as a marker of the late endosomal/lysosomal compartment (Saftig *et al.*, 2010) [48]. LAMP1 and LAMP2 together constitute a considerable proportion of lysosomal membrane proteins and participate in maintenance of lysosomal membrane integrity, autophagy and lysosomal homeostasis (Eskelinen, 2006) [30]. LAMP1 abundance is influenced by lysosomal biogenesis, trafficking, turnover and membrane composition (Eskelinen, 2006) [30]. In the present study, scopolamine significantly decreased both glycosylated and non-glycosylated LAMP1 protein expression in the prefrontal cortex. This decreased LAMP1 expression cannot be interpreted as evidence of a reduction in lysosome number, but when considered together with the reduced LysoTracker fluorescence observed in the cellular glial model, their combined alteration supports the presence of a broader disturbance of the lysosomal compartment. Upon scopolamine challenge, previous studies have shown that dysfunction or

imbalance in lysosomal pathways causes neurodegenerative processes (Drobny *et al.*, 2022) [27]. Thus, the reduction in LAMP1 observed following scopolamine challenge supports the concept that cholinergic disruption is accompanied by molecular remodeling of the lysosomal membrane system.

The present study showed that Cat B and Cat D expression were decreased following scopolamine challenge. Lysosomal cathepsins are important components of intracellular proteolysis and contribute to degradation of proteins delivered to lysosomes by endocytosis and autophagy (Saftig *et al.*, 2009) [31]. Cat B is a cysteine protease that participates in lysosomal protein degradation and several cellular regulatory processes (Wang *et al.*, 2023) [49]. Therefore, the reduction in Cat B observed in the present study indicates alteration of one component of the lysosomal proteolytic machinery. Cat D is a major lysosomal aspartyl protease highly relevant to neuronal proteostasis. It participates in degradation of damaged, oxidized and aggregation-prone proteins, and alterations in Cat D have been implicated in several neurodegenerative conditions (Vidoni *et al.*, 2016) [33]. Disturbances in cathepsin expression, localization, maturation or enzymatic activity can alter cellular proteostasis and are increasingly associated with neurodegenerative mechanisms (Drobny *et al.*, 2022; Vidoni *et al.*, 2016; Lewandowski *et al.*, 2025; Mijanovic *et al.*, 2021) [27, 33, 35, 50]. However, protein expression alone does not provide a direct measure of enzymatic activity, since Cat B function additionally depends on maturation, localization and lysosomal pH (Drobny *et al.*, 2022) [27]. Cat B and Cat D are synthesized as inactive precursor proteins, pro-Cat B and pro-Cat D, respectively, which undergo proteolytic processing within the acidic lysosomal compartment to generate their mature, enzymatically active forms (Nixon *et al.*, 2000; Ni *et al.*, 2022) [51, 52]. The conversion of pro-Cat B into mature Cat B involves removal of the propeptide followed by processing into the mature heavy- and light-chain forms, whereas pro-Cat D undergoes sequential cleavage to generate the mature heavy- and light-chain cathepsin D (Hartley *et al.*, 2023) [53]. In the present study, the detection of alterations in the expression of Cat B and Cat D forms may indicate dysregulated lysosomal proteolysis associated with neurodegenerative disease. Evidence demonstrates that deficiency or impairment of one cathepsin can produce altered expression or activity of other cathepsins to maintain cellular degradative capacity (Vidoni *et al.*, 2016; Mijanovic *et al.*, 2021) [33, 50]. However, this interpretation will require direct assessment of enzymatic activity and remains a future prospect of the study. The decreased expression of Cat B and Cat D however indicates that scopolamine suppresses the entire lysosomal proteolytic system in pre-frontal cortex.

TFEB is a master transcriptional regulator of a coordinated lysosomal gene network termed the CLEAR network. This network includes a broad group of genes involved in lysosomal biogenesis, hydrolase activity, autophagy and intracellular substrate degradation (Sardiello *et al.*, 2009; Palmieri *et al.*, 2011; Settembre *et al.*, 2011) [36, 37, 38]. TFEB activity is strongly regulated by phosphorylation and subcellular localization. Phosphorylated TFEB is predominantly retained in the cytoplasm, whereas dephosphorylation permits nuclear translocation and transcriptional activation of its target genes (Settembre *et al.*, 2011; Shariq *et al.*, 2024; Napolitano *et al.*, 2018) [38, 40, 54]. Under appropriate stimuli, TFEB can be dephosphorylated and translocated to the nucleus to activate CLEAR-network genes (Shariq *et al.*, 2024; Napolitano *et al.*, 2018) [40, 54]. In the present study, only total TFEB protein expression was assessed in the prefrontal cortex and this was significantly increased following scopolamine challenge. An important distinction however needs to be made between increased TFEB protein expression and functional TFEB activation since phosphorylated TFEB was not measured in the present study. Herein the increased TFEB expression in prefrontal cortex of scopolamine challenged mice occurred simultaneously with alterations in LAMP1 and Cat B and D and may be regarded as an attempted compensatory response to lysosomal stress. Assessment of nuclear versus cytoplasmic TFEB, TFEB phosphorylation status and transcription of CLEAR-network genes is required to determine whether the increased TFEB protein observed

herein is functionally active. This remains the future prospect of the study.

The overall pattern of LAMP1↓, Cat B↓, Cat D↓ and TFEB↑ as observed in this study does not support a simple model of uniform lysosomal suppression following scopolamine challenge. Rather, these findings suggest the presence of lysosomal stress accompanied by differential remodeling of the lysosomal network, with activation of compensatory TFEB-mediated responses despite reduced lysosomal markers and protease expression.

Future studies examining lysosomal pH, autophagic flux, cathepsin enzymatic activity and maturation, TFEB nuclear translocation, and expression of CLEAR-network target genes will be required to determine the precise functional consequences of these molecular alterations.

5. Conclusion

The present study demonstrates that scopolamine can produce an early alteration of the acidic vesicles compartment in glial cells. In vivo, scopolamine produces a distinct lysosomal molecular profile characterized by reduced LAMP1 and Cat B and D, expressions and increased TFEB expression in the mouse pre-frontal cortex. The findings support a model of dysregulated lysosomal homeostasis by scopolamine characterized by selective impairment and compensatory remodeling. The findings identify the lysosomal system as a previously underexplored component of scopolamine-associated cellular stress and suggest that altered acidic vesicular homeostasis and lysosomal proteostasis may occur with sustained cholinergic disruption.

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