Fusion of lysosomes with secretory organelles leads to uncontrolled exocytosis in the lysosomal storage disease mucolipidosis type IV

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Abstract

Mutations in TRPML1 cause the lysosomal storage disease mucolipidosis type IV (MLIV). The role of TRPML1 in cell function and how the mutations cause the disease are not well understood. Most studies focus on the role of TRPML1 in constitutive membrane trafficking to and from the lysosomes. However, this cannot explain impaired neuromuscular and secretory cell functions that mediate regulated exocytosis. Here, we analyzed several forms of regulated exocytosis in a mouse model of MLIV and, opposite to expectations, we found enhanced exocytosis in secretory glands due to enlargement of secretory granules in part due to fusion with lysosomes. Preliminary exploration of synaptic vesicle size, spontaneous mEPSCs, and glutamate secretion in neurons provided further evidence for enhanced exocytosis that was rescued by re-expression of TRPML1 in neurons. These features were not observed in Niemann–Pick type CI. These findings suggest that TRPML1 may guard against pathological fusion of lysosomes with secretory organelles and suggest a new approach toward developing treatment for MLIV.

Keywords exocytosis; lysosomes; secretory organelles; TRPML1 channel

Introduction

Mucolipidosis type IV (MLIV, MIM 252650) is an inherited lysosomal storage disease (LSD) characterized by neurodegenerative disorders with corneal opacity, achlorhydria, and impaired neuromuscular junctions [1]. MLIV is caused by mutations in the lysosomal TRP channel TRPML1 [2], rather than loss of specific lysosomal hydrolytic activity and altered lysosomal metabolism, typical of other LSDs. TRPML1 is a member of the superfamily of TRP channels and functions as a nonselective Ca2+-permeable cation channel [3]. TRPML1 is activated by the endolysosomal lipid PI(3,5)P2 [4] and participates in the regulation of lysosomal pH [5,6] and in Ca2+ release from lysosomes [7]. However, how mutations or deletion of TRPML1 in patients cause the disease is not well understood.

Morphological and biochemical studies of various tissues obtained from patients with MLIV showed cytoplasmic accumulation of inclusion bodies with multiple lamellar membranes [8,9], and storage of lipids and proteins [10,11]. These observations led to examining the role of TRPML1 in membrane trafficking into and from the lysosomes. Knockdown or deletion of TRPML1 were suggested to cause delayed exit of stored material from the lysosomes [12], perhaps by altering lysosomal exocytosis [13]. Recent interesting studies demonstrated a role of TRPML1 in phagocytosis of large particles, whereby activation of TRPML1 by PI(3,5)P2 resulted in lysosomal Ca2+ release, delivering the lysosomes to sites of nascent phagosomes to promote phagocytic ingestion of large extracellular particles such as apoptotic cells [14]. Similarly, TRPML1 is required for lysosomal-dependent membrane repair [15]. Deletion of TRPML1 results in accumulated damage and skeletal...
muscle wasting and weakness due to impaired membrane repair [15]. These functions of TRPML1 are attributed mostly to altered constitutive membrane trafficking that involves the lysosomes and occur on a time scale of many minutes to hours.

Another important cellular membrane trafficking is regulated exocytosis that occurs on a time scale of milliseconds. Whether and how altered lysosomal function due to deletion of TRPML1 affects regulated exocytosis to affect neuromuscular junctions and the function of secretory glands is unknown. Remarkably, in spite of the prevalent neurodegeneration in all LSDs and the importance of regulated exocytosis in neuronal function, how regulated exocytosis is altered in any LSD has not been investigated. A priori, because of the impaired constitutive membrane trafficking, it is generally expected that all cellular activities involving membrane traffic are inhibited in LSDs. The exact opposite is found here, revealing an unexpected function of TRPML1 in lysosomal function. In the present studies, we examined how impaired lysosomal functions in MLIV and in Niemann–Pick type C1 (NPC1) affect multiple forms of regulated exocytosis in several tissues. We discovered that deletion of Trpml1 results in a marked augmentation of all forms of regulated exocytosis likely due to fusion of lysosomes with secretory organelles to enlarge their size. Excessive exocytosis of digestive enzymes by the pancreas results in chronic pancreatitis. In addition, increased spontaneous mEPSCs and exocytosis of glutamate by Trpml1−/− neurons were observed. Excessive unregulated exocytosis was unique to MLIV and was not observed in NPC1. These findings indicate that a major role of TRPML1 is to guard against unintended, pathological fusion of the lysosomes with other intracellular organelles that in secretory cells include the secretory organelles. These findings have significant implication to understanding and treating MLIV.

Results

Slow progress of neuromuscular deficiency is unique to MLIV [1]. It is generally assumed that impaired neuromuscular function is due to impaired membrane trafficking that more severely affects neurons [16,17]. Moreover, it was shown recently that skeletal muscle repair is defective in Trpml1−/− mice due to impaired trafficking of lysosomes to the area of membrane damage [15]. However, excessive regulated exocytosis and rapid exhaustion of neurotransmitters can impair neuromuscular function and be a major cause of the neurodegeneration in MLIV. Remarkably, in spite of its importance in every physiological function and of its association with rapid membrane trafficking and fusion/tission events, regulated exocytosis has not been evaluated in MLIV or any other LSD. Here, we examined several forms of regulated exocytosis in mouse models of the lysosomal storage diseases MLIV and Niemann–Pick type C1.

Uncontrolled Ca2+-dependent exocytosis in Trpml1−/− mice

Regulated exocytosis by pancreatic acini is the classical model used to study Ca2+-stimulated exocytosis of secretory granules in non-excitable cells [18]. First, we examined the effect of deleting Trpml1 on receptor-evoked Ca2+ signaling in pancreatic acinar cells. Figure 1A shows that deletion of Trpml1 reduced the response of acini to stimulation by low concentrations of the secretagogue cholecystokinin (CCK), as reflected in reduced Ca2+ oscillations frequency. The response to low CCK concentration involves generation of NAADP and activation of TPC2, which resides at the lysosomes together with TRPML1 [19] and mediates the NAADP response in pancreatic acini [20]. It is likely that the CCK response releases Ca2+ from the lysosomal compartment that requires the function of TRPML1. At higher CCK concentrations (0.1–10 nM) when IP3 generation dominates the response, cell stimulation resulted in a biphasic peak-plateau response, at which no difference between wild-type and Trpml1−/− acini could be resolved (Fig 1B).

Regulated exocytosis by pancreatic acini occurs in two phases, a rapid Ca2+-dependent phase that is completed in < 5 min and is followed by a PKC-stimulated phase that is augmented by Ca2+ [21]. Impaired Ca2+ signaling in Trpml1−/− cells was expected to result in impaired exocytosis. Figure 1C shows that this was the case for the first phase at CCK concentrations between 0.3 and 3 pM measured during 5 min of stimulation. However, no difference was observed at 10 pM CCK in acini stimulated only for 5 min and, unexpectedly, an enhanced exocytosis was observed at the optimal physiological concentrations of 10–100 pM CCK measured during 30-min stimulation (Fig 1D and E).

In an attempt to understand the aberrant enhanced regulated exocytosis in spite of reduced Ca2+ signals, we determined the localization of secretory granules, amylase content, the actin cytoskeleton that acts as a barrier for regulated exocytosis [22], and the polarized localization of Rab5 and Rab6, which regulate organellar traffic [23]. These parameters are all unchanged in pancreatic (Fig EV1A and C) and parotid acini (Fig EV1B and C). In addition, although collapsing the acidic lysosomal pH by treatment with NH4+ reduced exocytosis in all cell types, it did not change the difference in CCK-evoked Ca2+ oscillations (Fig EV1D–F) or exocytosis by pancreatic (Fig EV1G) or by parotid (Fig EV1H) wild-type and Trpml1−/− acini.

Since the secretory granules and lysosomes are in close proximity in polarized secretory cells, we asked whether deletion of Trpml1 may have resulted in unintended pathological fusion of secretory granules with the lysosomes in a hybrid organelle and in granules self-fusion. We used multiple independent assays to test this possibility. First, we analyzed the size of secretory granules by TEM. The images and size analysis (Fig 2A) reveal a remarkable enlargement of pancreatic acini secretory granules in Trpml1−/− mice. Next, we used four independent assays to examine interaction between lysosomes and secretory granules. We reasoned that fusion of lysosomes and secretory granules should increase co-localization and association of their specific markers. Staining with the granules marker amylase and the lysosomal marker LAMP1 in fixed acini showed about 10-fold increased co-localization (Fig 2B). In live acini, labeling the lysosomes with lysiotracker revealed accumulation of lysosomes in the apical pole of Trpml1−/− pancreatic acini, increased overlap of lysosomes with secretory granule area, and the presence of high numbers of large vesicles that may represent hybrid organelles (Fig 2C). Biochemical evidence for interaction between the granules and lysosomes was obtained by measuring co-immunoprecipitation (Co-IP) of the lysosomal LAMP1 and two secretory granules markers VAMP8 [24] and Rab27b [25]. Figure 2D shows a marked increase in the Co-IP of the markers in pancreatic acini from Trpml1−/− mice. Finally, we determined if stimulation of exocytosis resulted in concomitant release of
digestive enzymes and lysosomal content by measuring CCK-stimulated exocytosis of lysosomal acid phosphatase (AP). Figure 2E shows that CCK-stimulated Trpml1/C0 pancreatic acini mediate large exocytosis of AP, indicating coincident release of lysosomal and granular contents.

Taken together, the multiple assays in Figure 2 strongly suggest fusion of lysosomes with secretory granules. This conclusion is further supported by the in vivo consequence of this fusion. Fusion of lysosomes with secretory granules and activation of digestive enzymes within the pancreas by Cathepsin B-mediated cleavage of proenzymes are the hallmark of pancreatitis [26,27]. Therefore, in Figure 3 we used a panel of assays to determine whether digestive enzymes are activated in the pancreas and the Trpml1/C0 mice develop pancreatitis. Figure 3A shows elevated plasma amylase, Figure 3B shows high pancreatic MPO levels indicative of inflammation in Trpml1/C0 mice, and Figure 3C shows edema and damage to the pancreatic parenchyma before induction of acute pancreatitis. Moreover, Figure 3D, E and F shows activation of trypsin, accumulation of ceramide at the granular area, and activation of autophagy, respectively, in the Trpml1/C0 mice that are as high as those observed in wild-type acini after induction of acute pancreatitis by stimulation with high concentration of CCK. Hence, all typical pancreatitis parameters tested in pancreatic tissue and in isolated acini indicate that the Trpml1/C0 mice have chronic pancreatitis, as expected from premature activation of digestive enzymes. This is most likely due to Cathepsin B-mediated cleavage and activation of the digestive enzymes that is observed in all forms of pancreatitis [26,27].

Uncontrolled cAMP-dependent exocytosis in Trpml1/C0 mice

The findings in Figures 1 and 2 raised the question of whether other forms of regulated exocytosis are altered in the Trpml1/C0 mice. To address this question, we analyzed fluid and amylase secretion in another model of exocytosis, that by salivary glands. Fluid secretion by salivary glands is Ca2+-driven fueled by activation of the Ca2+-activated Cl− and K+ channels [29], whereas regulated exocytosis is stimulated by the cAMP pathway [29]. The salivary glands cAMP...
pathway has PKA and Ecap1 components that independently stimulate regulated exocytosis \[30,31\]. In salivary glands, the Ca\(^{2+}\) signal is dominated by receptor-mediated Ca\(^{2+}\) influx \[32\], which is nearly normal in \(\text{Trpml1}^{-/-}\) cells (Fig 4A). Notably, the Ca\(^{2+}\)-stimulated fluid secretion (salivation) evoked by maximal (Fig 4B) or submaximal (Fig 4C) stimulation of the muscarinic receptors with pilocarpine is unaltered in \(\text{Trpml1}^{-/-}\) mice. On the other hand, exocytosis stimulated by isoproterenol, which acts through the cAMP pathways, is markedly augmented when measured in mice (Fig 4D) or in isolated acini at all times and isoproterenol concentrations (Fig 4E and F). Hence, deletion of \(\text{Trpml1}\) results in augmented regulated exocytosis, whether stimulated by the Ca\(^{2+}\) (pancreas) or by the cAMP (salivary glands) signaling pathways.

As in the pancreas, the secretory granules in parotid acini of \(\text{Trpml1}^{-/-}\) mice are markedly enlarged (Fig 5A and B). Deletion of \(\text{Trpml1}\) prominently increased the Co-IP of the lysosomal marker LAMP1 and the secretory granule markers VAMP8 and Rab27b in the parotid and submandibular glands (Fig 5C). Staining the lysosomes with lysotracker revealed the presence of numerous large vesicles in \(\text{Trpml1}^{-/-}\) acini (Fig 5D). Moreover, Figure 5E shows that deletion of \(\text{Trpml1}\) resulted in copious secretion of the lysosomal acid phosphatase into the saliva, supporting the notion that lysosomal and granules contents are mixed in the \(\text{Trpml1}^{-/-}\) fused organelles that simultaneously exocytose the lysosomal and granular contents.

Enhanced neuronal exocytosis in \(\text{Trpml1}^{-/-}\) mice

The most prominent MLIV phenotype is neurodegeneration \[1\], suggesting aberrant neuronal exocytosis. To test the role of TRPML1 in neurotransmission, we analyzed spontaneous mini Evoked Post Synaptic Currents (mEPSCs) from brain slices while inhibiting
action potentials with TTX and GABA receptors with Bicuculline [33]. Figure 6A and B shows example traces, Figure 6C shows current size distribution, and Figure 6D shows the frequency of the mEPSCs in wild-type and Trpml1/C0/C0 neurons. The basal mEPSC frequency in Trpml1/C0/C0 neurons was about 50% higher than in wild-type neurons (Fig 6D), and Trpml1/C0/C0 neurons showed higher number of large mEPSCs (Fig 6C, expanded portion). To exclude the possibility that the increased mEPSCs was due to elevated Ca2+, we measured basal and KCl-induced Ca2+ increase in brain slices. Figure 6E shows that basal Ca2+ was similar in the two groups, while KCl-mediated Ca2+ increase was slightly lower in Trpml1/C0/C0 neurons. The higher mEPSCs are present in neurons from 5-week-old mice, long before the development of noticeable neuronal symptoms, as was reported for muscle damage [15].

Next, we analyzed the state and number of synapses in the Trpml1/C0/C0 mice at age 2–3 months, before the development of the severe disease phenotype and death at 6–9 months. Figure 7A and B shows that synaptic vesicles (SVs) in wild-type synapses are fairly uniform in size. On the other hand, synapses in Trpml1/C0/C0 neurons tend to contain large number of enlarged SV (Fig 7B). The number of active synapses, as evaluated from the number of post-synaptic density (PSD) profiles, is reduced in the Trpml1/C0/C0 mice (arrows in Fig 7C and summary in Fig 7D), perhaps due to continuous use. This is further suggested by crowding of synaptosomes at the active
zone of Trpml1−/− neurons (Fig EV2A), reduced synaptosomes/synapse (Fig EV2B) with no significant change in PSD length (Fig EV2C), and the reduced level of PSD in all areas of the brain tested by Western blots (Fig EV3A and B).

To further assay the role of TRPML1 in neuronal exocytosis, cerebral cortical neurons were cultured and secretion of the neurotransmitter glutamate was determined. Excessive glutamate stimulation causes Ca2+ overload and neurotoxicity [34], which is common in neurodegenerative diseases [35]. Figures 7E and EV2D show significant increase in spontaneous glutamate release by Trpml1−/− neurons. KCl-stimulated exocytosis of glutamate was also enhanced in Trpml1−/− neurons (Figs 7E and EV2E). Of note, the expression of TRPML1 in the Trpml1−/− neurons was sufficient to rescue the aberrant exocytosis, reducing both the basal (Figs 7F and EV2F) and stimulated exocytosis (Figs 7F and EV2G) to the levels found in wild-type neurons. This indicates that reversing the damage to the lysosomes can rescue the disease phenotype. In agreement with the increased mEPSCs and basal glutamate secretion, Figure 7G and the blots in Figure EV3 show increased basal Co-IP of the v-SNARE VAMP2 and t-SNARE SNAP25 in the brains of the Trpml1−/− mice.

Enhanced exocytosis is specific for MLIV

To determine whether the enhanced exocytosis is specific for MLIV, we analyzed Ca2+ signaling and exocytosis in the Npc1−/− mice model. The results in Figures EV4 and EV5 indicate that enhanced exocytosis is specific for MLIV. Deletion of Npc1 reduced the amplitude of the Ca2+ spikes (Fig EV4A and B) and the receptor-stimulated Ca2+ influx (Fig EV4C). Npc1−/− pancreatic acini exhibited reduced Ca2+-driven exocytosis (Fig EV5A) and slightly reduced size of secretory granules (Fig EV5B). Npc1−/− neurons have normal SVs size (Fig EV5C). Finally, deletion of Npc1 inhibited Ca2+-driven fluid secretion (Fig EV5D) without affecting cAMP-driven exocytosis in salivary glands (Fig EV5E), although the size of the parotid acini granules was unchanged. Finally, the stimulated...
salivary glands did not secrete the lysosomal acid phosphatase. The activity of TRPML1 was reported to be reduced in other LSDs, including Npc1, to suggest a common pathology in LSD related to altered membrane traffic [7]. Our findings suggest that the pathology of MLIV is specific to this LSD.

Discussion

The lysosomes participate in many cellular functions by sensing and responding to cellular nutrients [36] and are a hub for membrane trafficking. Impaired lysosomal function, as occurs in lysosomal storage diseases, disturbs membrane traffic to and from the lysosomes [37]. Lysosomes can also undergo exocytosis, which plays a role in membrane repair [15], cell migration [38], and phagocytosis [14]. All these functions are mediated by the relatively slow constitutive trafficking pathway. Another form of membrane trafficking is regulated exocytosis in secretory cells and neurons. Although formation of secretory organelles involves trafficking through the secretory pathway, after their maturation the organelles are clustered close to the secretory membrane, being it the luminal membrane in secretory cells or the active zone in neurons. Notably, lysosomes do not have a role in regulated exocytosis. The present study reports that when lysosomal function is specifically altered by deletion of the lysosomal channel TRPML1, regulated exocytosis becomes uncontrolled.

Regulated exocytosis controls rapid responses to changes in the physiological status and is particularly active in neuronal system, making it more vulnerable to uncontrolled exocytosis. The present studies in secretory glands indicate that a key role of Trpml1 is to guard against unintended, pathological fusion of lysosomes with other intracellular organelles. That this function is specific to TRPML1 is shown by the rescue of normal exocytosis by the expression of TRPML1 in Trpml1/C0/C0 neurons. The major

Figure 5. Enlarged salivary glands secretory granules, association of lysosomes and secretory granules, and secretion of the lysosomal acid phosphatase in Trpml1/C0/C0 mice.

A, B Example TEM images (A) and granules size distribution (B) in parotid glands determined from 10 images obtained from three wild-type mice and 14 images obtained from 4 Trpml1/C0/C0 mice.

C Co-IP of the lysosomal LAMP1 and the secretory granule proteins VAMP8 and Rab27b. Input control is actin.

D Acini isolated from the parotid glands of wild-type and Trpml1/C0/C0 mice were incubated with mitotracker (green) and lysotracker (red). The large vesicles in the secretory granules/lysosomal area are marked by white arrows. The average number of enlarged profiles is given in the graph and obtained from three acinar preparations of each line (mean ± s.e.m.).

E Time course (left) and averaged 10 min secretion (right) of the lysosomal marker acid phosphatase in the saliva collected from wild-type (black) and Trpml1/C0/C0 mice (red) stimulated with 1 mg/kg pilocarpine and 0.6 mg/kg isoproterenol. The mean ± s.e.m. is from four mice of each line.

Source data are available online for this figure.
organelles in secretory cells are the highly fusogenic secretory granules containing the exocytosed material, which appear to be a major target of the aberrant fusion with lysosomes. The present work provides multiple independent evidence to suggest fusion between secretory granules and the lysosomes. First is the marked enlargement of the granules; second is the co-localization of granules and lysosomal markers both in fixed and in live acini; third is the Co-IP of granules and lysosomal markers; fourth is the concomitant agonist-stimulated secretion of granules and lysosomal content to the luminal space; and fifth and probably the strongest evidence is the development of pancreatitis in Trpml1 mice that includes intracellular activation of trypsin. It is amply established that intracellular activation of trypsin is by lysosomal cathepsin B [26,27]. This causes marked increase in all forms of regulated exocytosis, Ca2+-driven and cAMP-driven, in the Trpml1 mice. In addition to uncontrolled exocytosis, fusion of lysosomes and secretory organelles results in secretion of harmful lysosomal proteases to the luminal and perhaps the neuronal external space.

Localization of secretory granules and lysosomal markers, like LAMP1, can also take place in newly synthesized granules, which can lead to a constitutive-like secretion and secretion by the so-called minor regulated pathways [39,40]. However, this is unlikely to be of major contribution to the uncontrolled enhanced exocytosis in Trpml1 cells since the two minor pathways originate from small vesicle that move through intermediate endosomes [41], whereas the granules in the Trpml1 mice are markedly enlarged. Additionally, we observed trypsin activation in unstimulated Trpml1 pancreatic acini, which can never occur in the immature granules. Activation of trypsin from trypsinogen requires proteolysis within the acini that is mediated by lysosomal proteases [26,27]. Moreover, the minor pathways contribute < 5% of the secreted material, whereas at physiological receptor stimulation, exocytosis is nearly doubled in the Trpml1 cells, a level that is many folds higher than the capacity of the minor pathways. Hence, we conclude that aberrant fusion of lysosomes and secretory granules is the most likely cause of the runaway exocytosis.

**Figure 6.** mEPSCs and [Ca²⁺], in brain slices.

A, B Example traces of mEPSCs recorded from wild-type (A) and Trpml1 (B) mice brain slices as detailed in Materials and Methods. The lower traces in each panel show the portion of the traces marked by dotted boxes in an expanded time scale.

C Histogram distribution of the events at each amplitude. The lower graph shows the distribution of the higher amplitude mEPSCs.

D The frequency of mEPSCs in each line.

E The Ca²⁺ signal in response to depolarization with 30 mM KCl in wild-type and Trpml1 slices.

Data information: The results in (C, D) are the mean ± s.e.m. of eight experiments each obtained from four mice of each line. The results in (E) are the mean ± s.e.m. of 6 and 5 experiments with wild-type and Trpml1 slices, respectively.
Our preliminary exploration of synaptic vesicle size and exocytosis provides evidence for interesting changes. Although these changes are compelling, the data are too preliminary for a mechanistic interpretation and firm conclusions. The increase in spontaneous mEPSC and glutamate release may lead to high basal level of glutamate in the neuronal space. The high basal glutamate exocytosis can be the result of the increased basal interaction of the v- and t-SNARE in Trpml1−/− brains. Long exposure to glutamate is neurotoxic [34,35] and may cause the severe neurodegeneration and impaired neuromuscular junction in MLIV. Glutamate neurotoxicity and synaptic exhaustion can contribute to the reduced number of active synapses and reduced level of PSD in the brain of Trpml1−/− mice and contribute to the neuronal deficiency in MLIV patients.

The uncontrolled excessive exocytosis is specific to MLIV and was not observed in the well-characterized lysosomal storage disease NPC1. Hence, the phenotype reported here is not the consequence of lysosomal storage, but more likely due to loss of function of TRPML1. This is supported by the rescue of the exocytosis phenotype by the expression of TRPML1 in Trpml1−/− neurons. It follows that TRPML1 plays a central role in the many lysosomal fusion reactions, with which a major function of TRPML1 is to determine the specificity of fusion. At this time, it is not known how TRPML1 could carry out this role. One possibility is that TRPML1 supplies the Ca2+ essential for lysosomal fusion [4,7], with TRPML1 function regulated by specific interaction of the lysosomes with particular intracellular organelles or the plasma membrane. Another possibility is the function of TRPML1 in regulation of lysosomal pH. In the absence of TRPML1, the lysosomes are more acidic [5,6] and lysosomal pH plays a key regulatory role in lysosomal fusions [42]. It is possible that in the absence of TRPML1, continued leakage of Ca2+ from the acidic lysosome or enhanced fusogenicity by the

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Impaired synaptic vesicle size, number of PSD, and exocytosis in cortical neurons.

A Example TEM images of synapses in wild-type and Trpml1−/− mice brain cortex.

B The distribution of synaptic vesicle size determined in 10 and 14 images obtained from 3 wild-type and 3 Trpml1−/− mice, respectively.

C Example TEM images at low magnification of wild-type and Trpml1−/− mouse brain cortical slices with the dark PSD profiles marked by white arrows.

D Average number of PSD/μm² analyzed in at least 10 images obtained from three mice of each line.

E Glutamate levels and secretion by cultured cerebral cortical neurons. Total glutamate measured in non-stimulated wild-type (black) and Trpml1−/− (green) neurons and neurons stimulated by depolarization with 30 mM KCl (blue and red).

F Rescue of glutamate levels and secretion by the expression of TRPML1 in cultured cerebral cortical neurons. Total glutamate measured in non-stimulated Trpml1−/− neurons (green) and Trpml1−/− neurons transfected with TRPML1 (black) and untransfected (red) and TRPML1-transfected (blue) Trpml1−/− neurons stimulated by depolarization with 30 mM KCl.

G Averaged Co-IP of SNAP25 and VAMP2 in three brain regions of wild-type and Trpml1−/− mice. The images of all blots are given in Figure EV4.

Data information: Results in (D–G) are given as the mean ± s.e.m. *P < 0.05.
over-acidic lysosomes results in uncontrolled fusion with other organelles. In secretory cells with a dedicated role in regulated exocytosis, this leads to exposure of the secretory granules to lysosomal enzymes and uncontrolled regulated exocytosis.

**Materials and Methods**

**Animals**

All protocols used with the mice have been approved by the NIH animal use committee and by the Thomas Jefferson Institutional Animal Care and Use Committee. The Trpml1−/− mice [43] and Npc1−/− mice [44] have been described before.

**Isolation of pancreatic and parotid acini**

Pancreatic and parotid acini were prepared from 4- to 6-month-old mice as described previously [45]. Mice were sacrificed by inhalation of CO₂, and the pancreas or parotid glands were quickly removed into an ice-cold buffer containing (mM) the following: 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.4), 0.1% bovine serum albumin, 0.1% pyruvic acid, and 0.02% soybean trypsin inhibitor (solution A). The tissues were minced finely and were digested with collagenase P (0.25 mg/ml) dissolved in the buffered solution. After washing twice, the acini were suspended in the standard buffer and were kept on ice until use.

**Neuronal culture**

Cerebral cortical neurons were dissociated and cultured from cerebral cortex, as previously described [46]. Briefly, the mice were euthanized by cervical dislocation; the brains were quickly removed and immersed in ice-cold Hanks balanced salt solution (Mediatech, Manassas, VA). The cerebral cortex was dissected and minced, and the cells were dissociated by enzymatic digestion with papain, followed by mechanical trituration. After centrifugation at 1,000 × g, fractions enriched in neurons were collected and resuspended in culture medium containing Neurobasal-A (Invitrogen), which promotes the survival of postnatal neurons, 1% Glutamax (Invitrogen), 2% penicillin-streptomycin-ampicillin B solution (Invitrogen), and 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cells were plated on round, 25-mm glass coverslips, previously coated with poly-L-lysine (Sigma-Aldrich) in 6-well plates. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. After 24 h, the cultured neurons were transferred to complete growth medium containing 10% fetal bovine serum. The mitotic inhibitor cytosine β-arabinoside furanoside (1 μM) (Sigma-Aldrich) was added to the culture to inhibit glial cell proliferation [47]. Cells were used for imaging after 2–4 days in culture.

**Measurement of salivary secretion**

Salivary collection was as we described before [32]. In brief, mice were anesthetized with a mixture of 75 mg/kg ketamine and 1 mg/kg dexmedetomidine and underwent endotracheal intubation to maintain an open airway path. Salivation was stimulated by I.P. delivery of the muscarinic agonist pilocarpine to stimulate fluid secretion and of the β adrenergic agonist isoproterenol to stimulate exocytosis. Saliva was collected under minimal vacuum into pre-weighted tubes. Saliva was collected every 5 min for 20–30 min and was normalized to body weight.

**Induction of acute pancreatitis**

Acute pancreatitis was induced by IP injection of mice with cerulein as detailed before [45]. To test vulnerability of the Trpml1−/− mice to induce acute pancreatitis, the mice were injected hourly only four times over the 4-h period. Two hours after the last injection (a total of 6 h), the mice were sacrificed for evaluation of serum amylase and edema.

**Measurement of [Ca²⁺]**

Acini were loaded with Fura2 by incubation with 2 μM Fura2/AM for 30 min at room temperature in solution A. Acini attached to cover glass that formed the bottom of a perfusion chamber were continually perfused with a warm, 37°C solution A without pyruvic acid and soybean trypsin inhibitor. Agonists were added to the perfusate, fluorescence was recorded at excitation wavelengths of 340 and 380 nm, and emitted light was collected and digitized with a cutoff filter at 510 nm. Collected images were analyzed using MetaFluor and presented as the change in 340/380 ratio.

**Determination of amylase release**

Samples of stimulated pancreatic acinar suspension were collected and centrifuged for 5 s at 1,000 × g and the supernatants collected to measure secreted amylase. To determine amylase content in saliva, whole saliva secreted in response to IP injection of pilocarpine and isoproterenol was collected. Amylase activity was measured with a Phadebas kit (Pharmacia & Upjohn, Cambridge, MA) following the manufacturer's instructions. Briefly, each sample was diluted in phosphate-buffered saline containing the amylase substrate and incubated for 5 min at 37°C in a shaking water bath. Reactions were stopped by the addition of 2 N NaOH to the mixture. After centrifugation at 14,000 × g for 5 min, the supernatant was collected and OD was measured at an absorbance wavelength of 620 nm.

**Measurement of acid phosphatase (AP) release**

AP activity was measured in secreted fluids with an AP assay kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s instructions. Briefly, each sample was diluted in citrate buffer containing 4-nitrophenyl phosphate, an AP substrate, and incubated for 20 min at 37°C. Reactions were terminated by the addition of 0.5 N NaOH to the mixture, and absorbance was measured at a wavelength of 405 nm.

**Determination of intracellular trypsin activation**

Intracellular trypsin activity was measured using the cell-permeable synthetic substrate rhodamine 110–(CBZ-Ile-Pro-Arg)2, as described before [32]. Isolated pancreatic acini were stimulated with or without the high concentration of 10 nM CCK8 for 60 min at 37°C. The cells were then washed in a solution containing (mM) 5 HEPES, 150
NaCl, 2 EDTA (pH 7.4), and 10 μM substrate. The cells were incubated in the same solution but without substrate for an additional 20 min to allow complete substrate hydrolysis. Bright field and fluorescence images were captured, and results were analyzed by counting the number of fluorescent cells.

**Co-immunoprecipitation**

Co-immunoprecipitation (Co-IP) and Western blot analysis were as described in [48]. Briefly, the tissues (pancreas, submandibular, and parotid glands) were isolated from three wild-type and three *Trpml1*−/− mice. The tissues in solution A were minced finely. The minced tissues were washed three times in PBS and then homogenized in glass homogenizer on ice in 1 ml of RIPA buffer containing protease inhibitors. The homogenate was centrifuged at 14,000 x g in Eppendorf tubes. The supernatant was collected and was used for Co-IP, with 100 μl of lysates containing the same amount of protein for all the samples that were incubated with 50 μl of Protein G Sepharose beads for 2 h to pre-clear the lysates. The lysates were centrifuged, and the beads were discarded. The cleared lysates were incubated with 1 μl Lamp1 antibody (0.1 mg/ml) overnight at 4°C, and then, 50 μl of Protein G Sepharose beads were added and the incubation continued for 4 h at 4°C. Then, the beads were washed four times with RIPA buffer containing protease inhibitors and proteins were released by incubation at 95°C for 20 min with 60 μl of Laemmli’s buffer containing β-mercaptoethanol. Samples (20 μl) were run in a denaturing gradient gel (4–12%) and blotted for Rab27b (1:500, BD biosciences) and VAMP8 (1:500, Sigma). The samples were also blotted for β-actin for normalizing the protein loads.

**Recording mEPSC from brain slices**

Horizontal brain slices (300 μm) that include the hippocampus, subiculum, and entorhinal cortex were cut using a vibrating blade microtome from 5-week-old wild-type and *Trpml1*−/− littersmates. Mice deeply anesthetized with isoflurane were decapitated, and the tissues in solution A were minced finely. The brains were dissected out in ice-cold solution containing (in mM) 95 NaCl, 25 NaHCO3, 25 glucose, 50 sucrose, 2.5 KCl, 1.25 NaH2PO4, 0.1 CaCl2, 3 MgCl2, 0.4 ascorbic acid, 3 myo-inositol, and 2 Na+ pyruvate and aerated with 95% O2/5% CO2. Slices were incubated for 30 min at 37°C and then held at room temperature (22–24°C) for experiments in a solution containing (in mM) the following: 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 25 glucose, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myo-inositol, 2 Na+ pyruvate, 25 NaHCO3, 0.001 TTX (to prevent action potentials), pH 7.4 and bubbled with 95% O2/5% CO2. Bicuculline (10 μM) was included in the bath solution to block GABA receptors. When KCl concentration was increased (30 mM), NaCl concentration was decreased to maintain osmolarity.

For recordings mEPSCs, the pipette (2–4 MΩ) contained (in mM) the following: 125 K-gluconate, 20 KCl, 4 MgATP, 10 sodium-phosphocreatine, 0.3 GTP, 10 HEPES, and 0.5 EGTA, pH 7.2, adjusted with KOH. Whole-cell patch-clamp recording was accomplished using an EPC-10 amplifier (HEKA) in voltage-clamp mode (holding potential −80 mV) from hippocampal slices. Neurons in the slices were visually identified with infrared video microscopy and differential interference contrast optics. Miniature AMPA EPSCs (mEPSCs) were recorded from dentate gyrus granule cells. Data were filtered at 2 kHz, digitized at 10 kHz, acquired online and analyzed off-line. The recorded mEPSCs were analyzed using Clampfit 10.

**Measurement of glutamate release**

Glutamate levels were detected using an enzymatic assay [49–51]. In the presence of glutamate, L-glutamic dehydrogenase (GDH) reduces 13-nicotinamide adenine dinucleotide (NAD+) to NADH, a product that fluoroses when excited with UV light. Provided that GDH and NAD+ are added to the saline in which neurons were bathed, any glutamate released in the medium can be detected as an increase in NADH fluorescence around secreted neurons. Neurons on coverslips were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TIE (Nikon Inc, Melville, NY). The microscope is equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera. During the experiments, the Perfect Focus System was activated. The neurons were pre-incubated with 1 mM NADP+ and 50 units of glutamate dehydrogenase for 5 min. All the experiments were performed at room temperature (20–23°C). The fluorescence (excitation at 340 nm and emission at 460 nm) was acquired and analyzed using NIS-Elements AR software (Nikon Inc.). For rescue experiments, neurons in primary culture were transfected either with RFP and empty vector or with RFP + HA-TRPML1 using Turbofectin 8. After 48 h, transfected neurons were identified fluorometrically by the red color and glutamate release was analyzed in *Trpml1*−/− neurons transfected with RFP alone of RFP and TRPML1.

**Immunofluorescence**

Isolated acini were fixed with 4% paraformaldehyde for 15 min and permeabilized with a mixture of 0.3% saponin and 0.2% Triton X-100 in phosphate-buffered saline for 15 min at room temperature. After incubation in blocking solution containing 5% goat serum, cells were stained by incubation with anti-keramide (1:50) overnight. The following day, each sample was probed with FITC-conjugated secondary antibodies. Images were collected using a confocal microscope and analyzed using MetaMorph software. Staining for amylase, Rab5, Rab6, and LAMP1 was by incubation with polyclonal anti-amylase (1:100), anti-Rab5 (1:100), anti-Rab6 (1:100), and anti-LAMP1 (1:250) antibodies overnight and probing with FITC-conjugated secondary antibodies. Actin was visualized by incubation with 10 μM phalloidin for 20 min at RT. All overlaps were analyzed using ImageJ and particle counting as outlined in the program instructions. In brief, using Photoshop, the green and red images were converted to gray, flattened, and the background adjusted. The images were then opened in ImageJ, which generates an RGB image and a B&W image with the colocalization. To calculate the area of the image occupied by the overlap, a threshold is set after which a binary image is obtained in which the overlap signal is displayed. The analyze particles function of the program is then used to obtain the number of particles and the overlap as percentage of the total image.

For visualizing lysosomes and mitochondria in live cells, pancreatic acini were incubated for 6 min with 1 μM each of mitotracker and lysotracker at 37°C and immediately imaged or kept on ice in
the same medium for use within 30 min. Staining of parotid acini was much more intense and less clustered at the apical pole. Test of several conditions showed that the clearest results were obtained when parotid acini were incubated for 30 min with 0.5 μM lysotracker and 0.5 μM mitotracker at 37°C in a shaking water bath. The acini were washed twice with ice-cold PBS, and confocal images were collected at RT.

Electron microscopy

All tissue samples were cut into 1–3 mm cubes, fixed for 48 h at 4°C in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and washed with cacodylate buffer three times. The tissues were fixed with 2% OsO4 for 2 h, washed again with 0.1 M cacodylate buffer three times, washed with water, and placed in 1% uranyl acetate for 1 h. The tissues were subsequently serially dehydrated in ethanol and propylene oxide and embedded in EMBed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections, approximately 80 nm, were obtained by utilizing the Leica ultracut-UCT ultramicrotome (Leica, Deerfield, IL, USA) and placed onto 300 mesh copper grids and stained with saturated uranyl acetate in 50% methanol and then with lead citrate. The grids were viewed in the JEM-1200EXII electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV, and images were recorded on the XR611M, mid-mounted, 10.5M pixel, CCD camera (Advanced Microscopy Techniques Corp, Danvers, MA, USA).

Statistics

All experiments were repeated at least three times, and the results are given as means ± s.e.m. Differences between the groups were analyzed for statistical significance by arc-sin transformation for percentage and overlap results and calculated t-test assuming equal variances. Square-root transformation was used for granule and synaptosomal size differences, and t-tests were performed assuming unequal variances. Comparisons between more than two groups (Fig 3) and the time course were analyzed by the Holm–Bonferroni method ([52,53]). For time courses and concentration dependencies, pairwise comparisons of the effect least squares means using Student’s t-tests with no multiplicity adjustment were performed and the P-values were then subjected to Holm–Bonferroni corrections. All transformations and statistical analysis were with JMP software. Differences between two groups were analyzed by unpaired t-test. In all cases, P < 0.05 or better was considered statistically significant.

Expanded View for this article is available online.

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Author contributions

SP, MA, MSK, CCB, AJ, M2, MB, and PMZ performed and analyzed the experiments; CAW and FDP provided essential material; MAE, EB, LGW, DMS, and SM designed and directed the studies; and SM drafted the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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