



# Secretion of ATP from Schwann cells through lysosomal exocytosis during Wallerian degeneration

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## ARTICLE INFO

### Article history:

Received 21 October 2012

Available online 7 November 2012

### Keywords:

ATP

Lysosomal exocytosis

Schwann cells

Wallerian degeneration

Secretory lysosomes

## ABSTRACT

The present study demonstrates that adenosine triphosphate (ATP) is released from Schwann cells through lysosomal exocytosis during Wallerian degeneration and in response to stimulation. In primary Schwann cell cultures, ATP was stored in lysosomal vesicles. ATP could then induce  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis. Among three stimulants of lysosomal exocytosis (glutamate,  $\text{NH}_4\text{Cl}$  and zymosan), only  $\text{NH}_4\text{Cl}$  was sufficient to induce ATP release from *ex vivo* sciatic nerve explants at 3 days *in vitro*. Lysosomal exocytosis inhibitors (metformin, chlorpromazine and vacuolin-1) reversed the effect of  $\text{NH}_4\text{Cl}$ -enhanced ATP release, replicating the state of explants treated with  $\text{NH}_4\text{Cl}$  in the absence of lysosomal exocytosis inhibitors. Furthermore, we observed ATP release through lysosomal exocytosis during Wallerian degeneration in sciatic explant cultures using the recently identified vesicular nucleotide transporter (VNUT). From these experiments, we conclude that the exocytosis of lysosomes in Schwann cells during Wallerian degeneration is  $\text{Ca}^{2+}$ -dependent, and that it induces ATP release from Schwann cells.

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## 1. Introduction

Extracellular adenosine triphosphate (ATP) has been shown to be a significant signaling molecule released from peripheral neurons and Schwann cells. This ATP plays an important role in chemical communication between several cells in the peripheral nervous system by acting as a neurotransmitter [1,2]. For example, as an axonal signal, extracellular ATP inhibits Schwann cell proliferation and differentiation during development [3]. Especially in Schwann cells, some chemicals, such as uridine triphosphate or glutamate, induce ATP secretion through  $\text{Ca}^{2+}$ -dependant exocytosis [4,5]. Extracellular ATP increases intracellular  $\text{Ca}^{2+}$  concentrations, and the increase in  $\text{Ca}^{2+}$  can trigger the release of ATP or amino acids through exocytosis [4,6,7]. Evidence for exocytosis in Schwann cells has also been provided by experiments in which ATP release was blocked by inhibitors of exocytosis that prevent the formation of vesicles from the Golgi complex or disrupt the delivery of vesicles [4]. However, which type of vesicles is involved in exocytic ATP release from Schwann cells remains to be elucidated.

Lysosomal activation is increased in Schwann cells after nerve injury and is involved in subsequent myelin degradation after nerve injury [8]. However, lysosomes can also function as secretory lysosomes during regulated exocytosis, but not in the degradation of some types of cell debris [9]. Previous studies have indicated that lysosomes may act as ATP delivery vesicles; large amounts of ATP are stored and released from astrocytes [10,11] and microg-

lia [12] through lysosomal exocytosis and non-adrenergic, non-cholinergic autonomic nerves also contain a considerable amount of ATP that is concentrated in lysosomal vesicles *in vivo* [13]. In Schwann cells, this lysosomal exocytosis contributes to axon regeneration [14]. Exocytosis of vesicle contents requires the fusion of opposing membrane layers. VAMP7, a member of the vesicular SNARE family, is highly involved in this process [15]. A previous study suggested that VAMP7 was required for  $\text{Ca}^{2+}$ -triggered lysosomal exocytosis and showed that the functional interaction between VAMP7 and synaptotagmin VII (SytVII) was necessary for lysosomal exocytosis in non-secretory cells [16]. SytVII is a member of the synaptotagmin family of  $\text{Ca}^{2+}$ -binding proteins and can be localized to lysosomes undergoing membrane fusion [17,18]. Thus, SytVII/VAMP7-positive vesicles function as  $\text{Ca}^{2+}$ -dependant secretory vesicles.

In this study, we investigated the role of ATP release in lysosomal exocytosis. We hypothesized that extracellular ATP induces lysosomal exocytosis in Schwann cells and ATP is released from lysosomal vesicles via the functional association of SytVII with VAMP7. Furthermore, we used sciatic nerve explants cultures to provide evidence that lysosomal VAMP7 is increased in Schwann cells during Wallerian degeneration.

## 2. Materials and methods

### 2.1. Materials

The primary antibodies used for immunostaining or western blotting detected SytVII and LAMP1 (Santa Cruz Biotechnology,

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Santa Cruz, USA). VAMP7 was obtained from Osenses Pty Ltd. (Kewick, Australia). VNUT was obtained from MBL Co., Ltd. (Woburn, USA). Alexa Fluor 488- and 594-conjugated secondary antibodies were purchased from Life Technologies (Grand Island, USA). ATP, metformin (Met), quinacrine dihydrochloride (Qui), vacuolin-1 (Vac) and chlorpromazine (CP) were obtained from Sigma (St Louis, USA).

## 2.2. Animals

All of the procedures were performed according to protocols approved by the Kyung Hee University Committee on Animal Research and followed the guidelines for the use of experimental animals established by The Korean Academy of Medical Science. Every effort was made to minimize animals suffering, and to reduce the number of animals used. Male Sprague–Dawley rats (6-weeks old) were housed with food and water available *ad libitum* in a temperature- ( $23 \pm 1^\circ\text{C}$ ) and humidity- (50%) controlled environment on a 12-h light/dark cycle.

## 2.3. Primary Schwann cell cultures

Cells were purified and cultured as described previously [19]. Briefly, after sciatic nerve axotomy to enhance the Schwann cell population, the rats were housed in plastic cages for 3 days. The sciatic nerves were removed aseptically and incubated in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HANK'S balanced salt solution containing 0.2% collagenase A (Roche Molecular Biochemicals, Nutley, USA) at  $37^\circ\text{C}$  for 2 h. After enzymatic digestion, the nerves were dissected by mechanical trituration with a pipette. The cell pellets obtained after centrifugation were re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin. Approximately, 20,000 cells/ $\text{cm}^2$  were seeded on a cover glass and allowed to grow for 2 days. To promote cell proliferation, 3 days after seeding, primary Schwann cells were cultured in the presence of forskolin (10  $\mu\text{M}$ , Calbiochem, San Diego, USA) and NGR-1 (200 ng/mL, R&D system, Minneapolis, USA).

## 2.4. Explant culture

Sciatic nerve explants were cultured as described previously [20]. The sciatic nerves of rats were removed, and the connective tissues surrounding the nerves detached using a stereomicroscope. The sciatic nerves were divided into 2–3 explants of 5 mm in length. The explants were cultured in DMEM containing penicillin–streptomycin and 10% FBS. Cultures were maintained at  $37^\circ\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . After being cultured, the sciatic explants and pieces of sciatic nerve removed following axotomy were fixed with 4% paraformaldehyde (PFA) overnight. The nerves were then cryoprotected in 30% sucrose for 2–3 days before being mounted in OTC medium and processed for immunostaining.

## 2.5. Immunofluorescence labeling

Primary Schwann cells or frozen nerve sections on slides were fixed in 4% PFA for 15 min. After washing three times with phosphate-buffered saline (PBS), the samples were permeabilized in ice-cold methanol for 10 min and then blocked with PBS containing 0.3% Triton X-100 (PBST) and 10% bovine serum albumin (BSA) for 1 h at room temperature (RT). Samples were incubated overnight with appropriate primary antibodies (1:1000) in PBS containing PBST at  $4^\circ\text{C}$  and washed three times with PBS. Next, samples were incubated with appropriate secondary antibodies (1:1000) for 1 h at RT. The slides were washed three times with

PBS, and coverslips were adhered to the slides with Gelmount (Bio-medex, Foster City, USA). The samples were analyzed using a laser scanning confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany).

## 2.6. Quinacrine staining

Quinacrine staining *in vitro* was performed by incubating sciatic nerve explants with Krebs–Ringer–Hepes (KRH: 125 mM NaCl, 5 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 2 mM  $\text{CaCl}_2$ , 6 mM glucose and 25 mM Hepes/NaOH, pH 7.4) containing 50  $\mu\text{M}$  quinacrine dihydrochloride for 30 min at  $37^\circ\text{C}$  as previously described [21]. During quinacrine staining, the samples were protected from light.

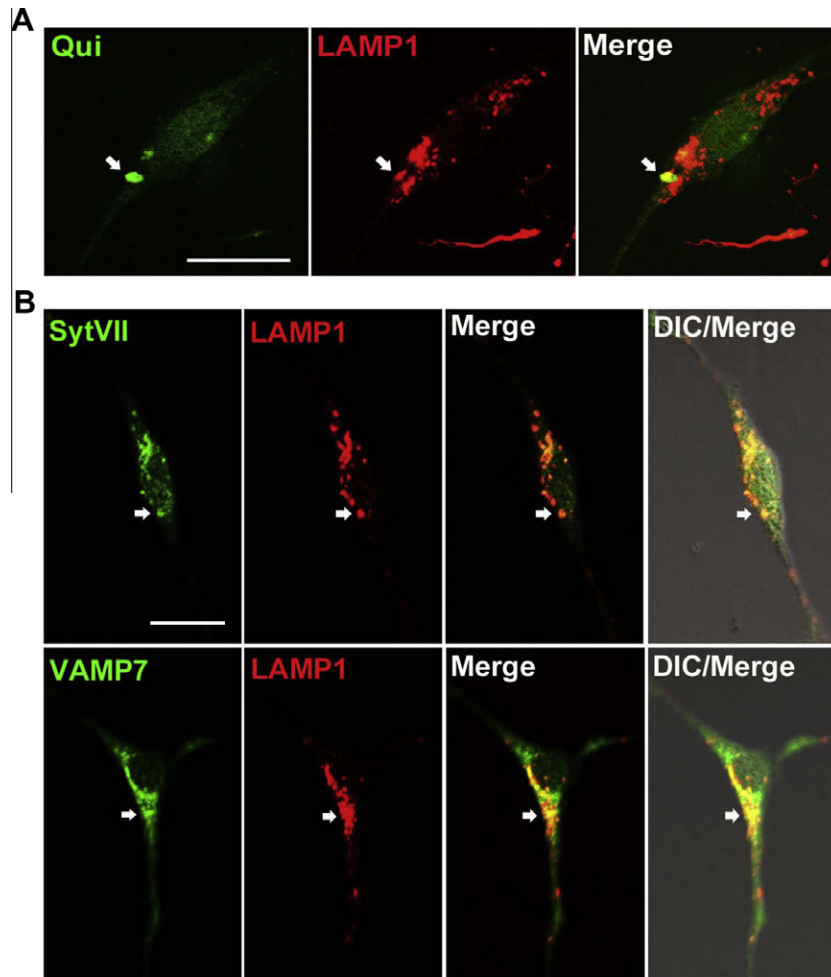
## 2.7. Extracellular ATP

Extracellular ATP was quantified using the luciferase–luciferin bioluminescence method (Promega, Madison, USA) and a luminometer (Berthold Technologies, Oak Ridge, USA) as described previously [22]. Briefly, glutamate,  $\text{NH}_4\text{Cl}$  and zymosan were added to the each sciatic explant media, and the supernatant was collected 3 days after chemical stimulation. Supernatant samples of 50  $\mu\text{L}$  were added to 50  $\mu\text{L}$  of excess ATP assay mix (1 mg/1 mL luciferin–luciferase).

## 3. Results and discussion

Previous studies have shown that ATP induces  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis in Schwann cells [7,12]. To identify whether intracellular ATP is stored in the lysosomal vesicles of Schwann cells after being treated with ATP to enhance lysosomal exocytosis, we first performed quinacrine staining to detect ATP. We then performed immunofluorescence staining using an antibody specific to LAMP1 in exocytosis-enhanced Schwann cell cultures treated with ATP. We found that incubation with quinacrine resulted in granule-like fluorescent spots. These quinacrine-stained spots were co-localized with lysosomal vesicles (Fig. 1A). This finding indicates that ATP is stored in lysosomal vesicles during exocytosis. Next, we used double-immunostaining with SytVII and VAMP7 antibodies to assess whether ATP-induced lysosomal exocytosis in primary Schwann cells is a  $\text{Ca}^{2+}$ -dependent process. Fig. 1B shows extensive overlap of LAMP1 staining with VAMP7 staining (upper panel) and SytVII staining (lower panel). Previous studies have shown that, in NRK cells, SytVII and VAMP7 are localized to LAMP1-positive lysosomes [16,23]. This finding suggests that ATP-induced lysosomal exocytosis in Schwann cells is a  $\text{Ca}^{2+}$ -dependent form of lysosomal exocytosis.

To confirm whether, during Wallerian degeneration, lysosomal exocytosis in Schwann cells induces ATP release from lysosomal vesicles after chemical stimulation, we quantified extracellular ATP in the *ex vivo* sciatic nerve explant model of degeneration using luciferase–luciferin bioluminescence system. Glutamate [5,10],  $\text{NH}_4\text{Cl}$  [24] and zymosan [25] are known to enhance lysosomal exocytosis in cell culture systems. Endogenous ATP was released from isolated explants upon stimulation with  $\text{NH}_4\text{Cl}$  but from neither glutamate nor zymogen (Fig. 2A). To determine whether  $\text{NH}_4\text{Cl}$ -induced ATP secretion is dependent on lysosomal exocytosis, we next repeated the experiment with the lysosomal exocytosis inhibitors metformin (Met) [26], chlorpromazine (CP) [27] and vacuolin-1 (Vac) [28] added to  $\text{NH}_4\text{Cl}$ -treated sciatic nerve explants. The extent of endogenous ATP release from the sciatic nerve explants was then measured. We found that  $\text{NH}_4\text{Cl}$ -induced ATP release from sciatic nerve explants was reduced by the



**Fig. 1.** ATP is stored in lysosomal vesicles associated with synaptotagmin VII and VAMP7. A. Quinacrine staining (Qui, green) and immunofluorescence labeling of LAMP1 (red) in Schwann cell primary cultures. Yellow staining indicates an overlap of green and red label and thus cell positively stained for both ATP and LAMP1. ATP-containing lysosomal vesicles (arrow) are present in Schwann cells. Scale bar = 10  $\mu$ m. B. Activation of synaptotagmin VII (SytVII) and VAMP7 is observed in LAMP1-positive vesicles (arrows). Top, double-immunofluorescence staining for SytVII (green) and LAMP1 (red) in primary Schwann cells. Bottom, primary Schwann cells co-immunostained with VAMP7 (green) and LAMP1 (red). Scale bar = 10  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

addition of the lysosomal exocytosis inhibitors Met and CP but not by Vac (Fig. 2B).

Next, we assessed  $\text{Ca}^{2+}$ -triggered lysosomal exocytosis in *ex vivo* sciatic explants during Wallerian degeneration using double-immunostaining for VAMP7 and LAMP1. Confocal microscopy images showed that labeling for VAMP7 and LAMP1 was co-localized during Wallerian degeneration (Fig. 3, top). We also observed co-localization of immunolabeling for VAMP7 and S100 (a Schwann cell marker) during Wallerian degeneration (Fig. 3, bottom).

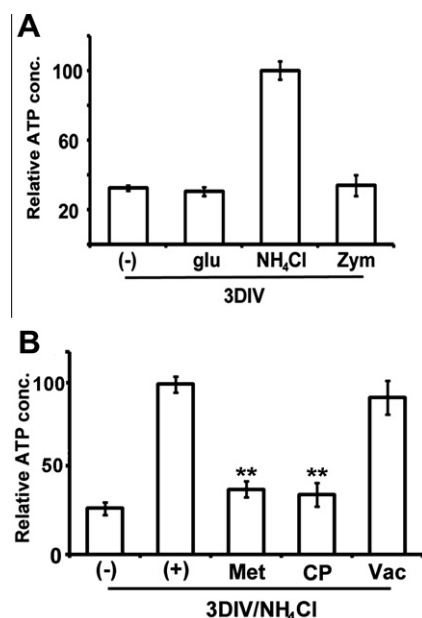
A previous study has shown that a vesicular nucleotide transporter (VNUT) has the capacity to transport ATP into vesicles [29]. To identify the mechanism of ATP release from Schwann cell lysosomes during Wallerian degeneration, we examined sections of *ex vivo* sciatic explants double-immunostained for VNUT and LAMP1. We confirmed that VNUT immunofluorescence labeling was localized to LAMP1-positive regions of sciatic explant sections during Wallerian degeneration (Fig. 4, top). Furthermore, we showed that immunolabeling for VNUT and S100 were co-localized in sciatic explant sections (Fig. 4, bottom). Altogether, these findings suggest that, in the *ex vivo* sciatic explant system, ATP release from Schwann cells occurs through  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis during Wallerian degeneration.

The role of ATP as a neurotransmitter is now firmly established. In the nervous system, extracellular ATP has been implicated as an

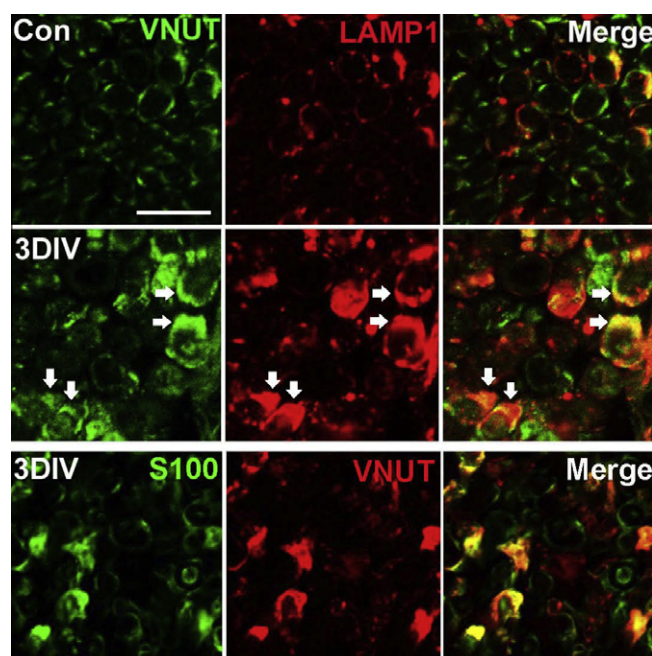
extracellular messenger for intercellular communication, such as glia-to-glia and glia-to-neuron interactions [1]. In the peripheral nervous system, Schwann cells, as a type of glial cell, have a multifaceted role in both the conduction of nerve impulses along axons and in nerve degeneration/regeneration. Thus, the identification of cellular processes that may lead to the release of ATP in Schwann cells is essential. However, the molecular mechanisms by which ATP is stored and released from vesicles in Schwann cells are largely unknown. Here, we demonstrated that  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis induces the secretion of ATP from Schwann cells during Wallerian degeneration.

Previous studies have shown that lysosomal vesicles contain abundant ATP and that ATP is released from microglia or astrocytes through lysosomal exocytosis in response to stimulation [10,12]. A growing body of evidence also indicates that Schwann cells can release ATP to the extracellular space and that the secretion of several proteins, such as lysosomal enzymes, is mediated by  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis in Schwann cells [3–7]. In this study, we identified the interaction between VNUT, SytVII, VAMP7 and lysosomal vesicles that is associated with exocytic ATP release from Schwann cell lysosomes during Wallerian degeneration. Several independent lines of evidence support this conclusion. First, we demonstrated in primary Schwann cell cultures that ATP is stored in Schwann cell lysosomal vesicles following ATP

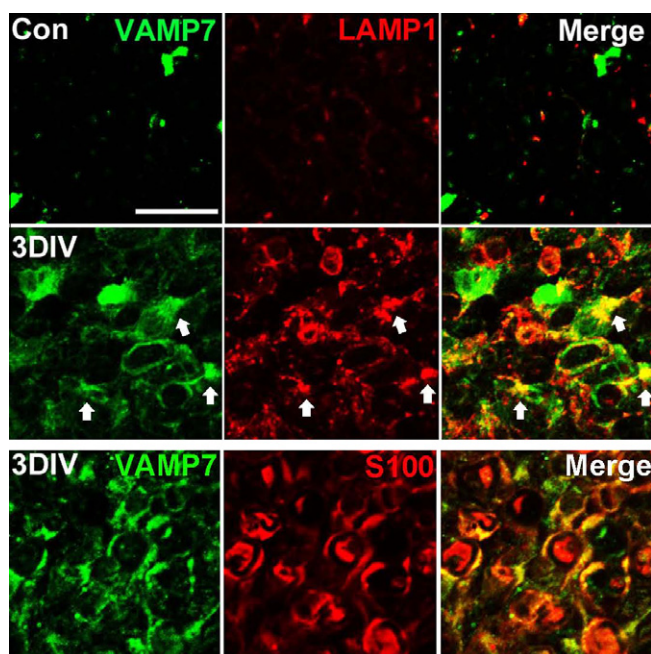




**Fig. 2.** Secretion of ATP is dependent on lysosomal exocytosis in *ex vivo* sciatic explants. **A.** Effects of the lysosomal exocytosis activator NH<sub>4</sub>Cl on ATP release in *ex vivo* explants. NH<sub>4</sub>Cl (4 mM) induced ATP release, while glutamate (glu, 1 mM) and zymosan (Zym, 200 µg/mL) did not significantly increase ATP release at 3 days *in vitro* (3DIV). **B.** Effects of lysosomal exocytosis inhibitors on NH<sub>4</sub>Cl-stimulated ATP release. The lysosomal exocytosis inhibitors, metformin (Met, 500 µM) and chlorpromazine (CP, 30 µM) significantly decreased the effects of NH<sub>4</sub>Cl. As a lysosomal exocytosis inhibitor, vacuolin-1 (Vac, 1 µM) had no effect on NH<sub>4</sub>Cl-stimulated ATP release at 3DIV. Statistical significance (\*\**p* < 0.01) was analyzed using the relative values of peak ATP release when incubated of lysosomal exocytosis inhibitors compared with incubation with NH<sub>4</sub>Cl alone.



**Fig. 4.** A vesicular nucleotide transporter is located in lysosomal vesicles. Top, confocal images of the vesicular nucleotide transporter (VNUT, green) and LAMP1 (red) immunofluorescence labeling in cross sections of rat sciatic explants. VNUT and LAMP1 activation were increased at 3DIV compared with the control. VNUT/LAMP1-double positive lysosomes are indicated by arrows. Bottom, cross sections of sciatic explants immunostained for S100 (green) and VNUT (red). Activation of VNUT is observed in S100-positive Schwann cells. Scale bar = 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** VAMP7 is required for Ca<sup>2+</sup>-dependent lysosomal exocytosis in Schwann cells. Top, cross sections of rat sciatic explants immunostained for VAMP7 (green) and LAMP1 (red). In the control (Con), VAMP7 and LAMP1 expression was clearly weak, but at 3DIV, VAMP7 and LAMP1 activation was increased. Arrows show lysosomes positive for VAMP7/LAMP1. Bottom, double immunostaining for VAMP7 (green) and S100 (red) was performed on cross sections of sciatic explants. Activation of VAMP7 is observed in S100-positive Schwann cells. Scale bar = 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment (Fig. 1A). We also showed that VAMP7 and SytVII are localized to lysosomal vesicles (Fig. 1B). Because VAMP7 is required for Ca<sup>2+</sup>-triggered lysosomal exocytosis [16] and lysosomal SytVII regulates Ca<sup>2+</sup>-triggered lysosomal exocytosis [15], these findings indicate that, in Schwann cells, lysosomal vesicles can contain ATP and function as secretory lysosomes that induce ATP release from Schwann cells. These processes are also associated with VAMP7 and SytVII, suggesting that ATP release occurs through Ca<sup>2+</sup>-dependent lysosomal exocytosis in Schwann cells.

Second, we also observed the secretion of ATP in *ex vivo* sciatic explant cultures. Among the enhancers of lysosomal exocytosis, glutamate, NH<sub>4</sub>Cl and zymosan, only NH<sub>4</sub>Cl could induce ATP release at 3 days *in vitro* (3DIV) during Wallerian degeneration. The inhibitors of lysosomal exocytosis Met and CP, but not Vac, were shown to inhibit the effect of NH<sub>4</sub>Cl on increasing ATP release (Fig. 2A and B). Interestingly, in confocal images of double-immunostained sciatic explants, we found co-localization of activated LAMP1 and VAMP7 during Wallerian degeneration. We also found that activation of VAMP7 occurred in Schwann cells (Fig. 3). In addition, we found that expression of VNUT, a vesicular nucleotide transporter, is increased during Wallerian degeneration and that VNUT co-localizes with lysosomal vesicles at 3DIV in Schwann cells (Fig. 4). VNUT has been identified as an ATP transporter in secretory vesicles. ATP is secreted by the fusion of intracellular vesicles that contain ATP through the action of VNUT with the plasma membrane [29]. These findings suggest that, during Wallerian degeneration, the increased expression of VNUT leads to enhance filling of lysosomal vesicles with ATP. ATP release then occurs through Ca<sup>2+</sup>-triggered lysosomal exocytosis in Schwann cells. However, because extracellular ATP acts as an axonal signal that inhibits the proliferation and differentiation of Schwann cells [3], the increase in extracellular ATP may be sufficient to inhibit Schwann cell de-differentiation and proliferation and to delay peripheral nerve

regeneration. Thus, to prevent this phenomenon, it seems likely that another mechanism, such as the swift degradation of ATP to adenosine by extracellular ectonucleotides [30], may be at work.

#### 4. Conclusion

In this study, we demonstrated that ATP is stored in lysosomal vesicles following stimulation lysosomal exocytosis activators. We also showed that the secretion of ATP from Schwann cells occurs through  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis during Wallerian degeneration. Thus, we propose that the regulation of lysosomal exocytosis in Schwann cells may be a potential target for novel therapeutic strategies for the treatment of peripheral neuropathies and nerve injury.

#### Acknowledgments

This work was supported by the Global Frontier Project grant (2012M3A6A4054932) of the National Research Foundation funded by the Ministry of Education, Science and Technology of Korea.

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