Possible ATP release through lysosomal exocytosis from primary sensory neurons

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ABSTRACT

The adenosine triphosphate (ATP) plays important roles under physiological and pathological conditions such as traumatic brain injury, neuroinflammation and neuropathic pain. In the present study, we set out to study the role of lysosomal vesicles on ATP release from the dorsal root ganglion neurons. We found that the lysosomal vesicles, which contain the quinacrine-positive fluorescence and express the vesicular nucleotide transporter (VNUT), were localized within the soma and growth cone of the cultured dorsal root ganglion neurons. In addition, the number of the quinacrine staining was decreased by application of lysosomal exocytosis activators, and this decrease was suppressed by the metformin and vacuolin-1, which suppressed lysosomal exocytosis. Thus, these findings suggest that ATP release via the lysosomal exocytosis may be one of the pathways for ATP release in response to stimulation.

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1. Introduction

To communicate with other cells, neurons employ transmitters that are released from vesicles at presynaptic terminals [1]. There is strong evidence for exocytotic vesicular release of adenosine triphosphate (ATP) from neurons [2]. In presynaptic terminals, ATP is transported into the synaptic vesicles [3], and the vesicular release of ATP occurs [4–7]. The role of ATP as a neurotransmitter of relevant purinergic signaling is established [8]. When the great auricular nerve is stimulated, ATP is liberated from stimulated sensory nerves [9]. ATP becomes a transmitter important for signaling injurious nociceptive information [10]. Although the ATP release via synaptic vesicle is well established, additional machineries to release ATP become evident [2].

Lysosome is an organelle, which functions to degrade proteins and organelles particularly in event of phagocytosis and autophagy, however recent studies demonstrated additional function of lysosome in the regulated exocytosis (secretory lysosomes) [11]. These secretory lysosomes are distinguished from conventional lysosomes by the ability to undergo regulated secretion [11]. A previous report indicated that non-adrenergic, non-cholinergic autonomic nerves contain a considerable amount of ATP concentrated in lysosomal vesicles *in vivo* [12]. Recent studies also support that large amounts of ATP is stored in lysosomes, and ATP is released from lysosomes in astrocytes [13,14] and microglia [15] through lysosomal exocytosis. Lysosomal vesicles are acidified by its H^+-ATPase [11], and lysosomal exocytosis can be triggered by chemicals that cause alkalinization of lysosomes [16]. Thus, pH regulation of lysosomal vesicles would determine the fate of lysosomal vesicles, 'secretory lysosomes or conventional lysosomes' in several cell types [17]. However, the behaviors of ATP through lysosomal exocytosis in neurons remain to be elucidated. Here we present that ATP is stored and released from lysosome in cultured dorsal root ganglia (DRG) neurons, and discuss the possible release of ATP through lysosomal exocytosis in the DRG neurons.

2. Materials and methods

All of the efforts were made to minimize animals suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Male Sprague–Dawley rats (6 weeks old) were housed with food and water available *ad libitum* in a temperature ($23 \pm 1 °C$) and humidity (50%) controlled environment on a 12/12 h light/dark cycle. All of the experimental procedures were performed according to the standard guideline for animal experiments of the Graduate School of Medicine, Nagoya University.

Dissociated DRG neuronal cultures were performed according to our previous study [18]. Lumbar DRG was removed aseptically from rats on postnatal day 0 and was incubated in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} containing 0.125% collagenase A (Roche, USA) and penicillin–streptomycin (PS) for 45 min at 37 °C followed by 2.5% trypsin treatment for 15 min. After enzymatic digestion, cells were dissected by mechanical trituration through pipetting and transferred into DMEM/F12 (Wako, Japan) medium containing 10% bovine serum and PS. Approximately,

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2000 cells were seeded on a cover glass coated with poly-D-lysine (PDL, 100 mg/mL; Sigma, USA) and laminin (100 µg/mL, Sigma, USA) and incubated for 14 h at 37 °C with 5% CO_2 atmosphere. Primary DRG neurons were incubated for 4 h in the presence of various lysosomal exocytosis agonist or antagonist; ATP (4 mM, Sigma, USA), chloroquine (20 µg/mL, Sigma, USA), metformin (500 µM, Sigma, USA) and vacuolin-1 (1 µM, Sigma, USA).

To visualize ATP-containing vesicles, we used quinacrine, fluorescent dye, and is generally used for detecting releasable ATP stores. Quinacrine staining *in vitro* was performed by incubating living cultured DRG neurons for 30 min at 37 °C with Krebs–Ringer–Hepes (KRH: 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 6 mM glucose, 25 mM Hepes/NaOH, pH 7.4) containing 50 µM quinacrine dihydrochloride (Sigma, USA) as previously described [19]. During quinacrine staining, slides and staining solution were protected from light. The quinacrine staining slides were observed under fluorescent microscope with excitation filter 450–490 nm (Carl Zeiss, Germany).

For immunofluorescent staining, the slides were blocked with PBS containing 0.3% Triton X-100 and 10% bovine serum albumin for 1 h. Samples were then incubated with appropriated primary antibody (anti-LAMP1; Santa Cruz, USA, VNUT; MBL, USA) for 1 h at 4 °C and washed three times with PBS. Next, the slides were incubated with Alexa 488- or Alexa 594-conjugated secondary antibody (1:1000, Invitrogen, USA) for 1 h at room temperature. Samples were then washed three times with PBS, and coverslips were adhered to glass slides with a mounting medium and viewed using a laser confocal microscope (LSM710, Carl Zeiss, Germany).

For quantification of extracellular ATP, we used a bioluminescence method using the luciferase–lucifrin system (Promega, USA) by luminometer (Berthold Technologies, USA) as described previously [20]. Briefly, a chemical was added to primary DRG culture medium, and supernatant was collected at 1 h after stimuli. 50 µL supernatant was added to 50 µL of excess ATP assay mix (1 mg/1 mL luciferin–luciferase).

Differences in the means between groups were statistically assessed using an analysis of variance followed by the Bonfererroni post hoc test. Differences were considered statistically significant at the $P < 0.05$, $P < 0.1$ and $P < 0.01$ level from three independent experiment.

3. Results and discussion

To visualize ATP-containing vesicles in DRG neurons, we applied quinacrine (Quin), an ATP binding chemical, to primary cultures of DRG neurons. Because Quin, an acridine derivative, has a very high affinity for ATP, Quin-staining has been used to identify intracellular ATP-enriched vesicles in several cell types [19,21–26]. Quin-positive staining was observed in vesicles in neuronal soma and the tip of elongating process of cultured DRG neurons (arrow), suggesting the presence of ATP-filled vesicles (Fig. 1A, top). In addition, LAMP1 (a lysosomal marker) had a distribution similar to that of ATP-filled vesicles in the DRG neuron (Fig. 1A). In the tips of elongating process, merged image showed a high degree of co-localization between the Quin staining and LAMP1. Although in the neuronal soma, the Quin staining did not always co-localize with the LAMP1 staining in high magnification images, indicating that not all lysosomal vesicles contained the Quin detectable level of ATP (Fig. 1A, bottom). Here, the Quin-positive fluorescence indicating ATP content was almost always observed within LAMP1-positive lysosomal membrane (Fig. 1B, arrow). Quin-positive lysosomal vesicles were preferentially located at the outer area of cell body of DRG neuron (Fig. 1B). The Quin-positive staining in the central region of cell body was associated with DNA in nucleus of the DRG neuron [27]. A previous study showed that

the vesicular nucleotide transporter (VNUT) function to transport ATP into intracellular vesicles, and VNUT could be a good marker for ATP containing vesicles [28]. We then assessed whether VNUT-positive vesicles contained Quin staining using immunofluorescent labeling for VNUT. We found that Quin-positive staining is observed in VNUT-positive vesicles in neuronal soma and the tip of elongating process of cultured DRG neurons (Fig. 2A, arrow), and high magnification images showed the colocalization of VNUT with LAMP1 or Quin staining (Fig. 2B). These results indicate that some lysosomal vesicles in the DRG neurons are capable of storing ATP.

We next examined a possibility that a treatment with chemicals, which trigger lysosomal exocytosis, could induce the release of ATP from DRG neurons. To identify ATP release from DRG neuron by lysosomal exocytosis, we have used ATP as a lysosomal exocytosis agonist [15], because an extracellular application of ATP elicits an increase in lysosmal pH, resulting in lysosomal exocytosis [29]. As shown in Fig. 3A, treatment with ATP reduced the number of the Quin-positive vesicles in DRG-neuronal soma. Another reagent the metformin (Met) was reported to acidify lysosomal compartments in rat microglia [30], and in addition Cerny et al. [31] reported that lysosomal exocytosis was blocked by the vacuolin-1 (Vac). We therefore have used Met and Vac as lysosomal exocytosis inhibitors to block the ATP-induced lysosomal exocytosis. Those inhibitors were significantly suppressed the decrease of the number of Quin-positive vesicles in DRG neurons pretreated with ATP (Fig. 2A and B). However, the number of LAMP1-positive vesicles were not changed after treatment of all ATP, Met and Vac (Fig. 3A). These findings suggest that the activation of lysosoaml exocytosis may be involved in the change of amount of lysosomal vesicles.

Lastly, to numerically identify ATP release through the lysosomal exocytosis in primary DRG neurons, we performed the quantification of extracellular ATP with a bioluminescence method using the luciferase–luciferin system. Glutamate, NH_4Cl and zymosan are known as lysosomal exocytosis activators in cell culture system [13,32–34]. There were significant ATP release from the isolated DRG neurons with zymosan and NH_4Cl, not glutamate (Fig. 4A), and the simultaneous application of the lysosomal exocytosis inhibitors, Met and Vac, significantly blocked zymosan-induced ATP release from DRG neurons (Fig. 4B).

Collectively, these results showed that the Ca^{2+}-dependent lysosomal exocytosis could elicit ATP release from the DRG neurons.

Previous studies demonstrated ATP release from astrocytes [13,14] and microglia [15] through lysosomal exocytosis *in vitro*. These studies account for the possibility that lysosomal exocytosis might be one of mechanism for vesicular ATP release. In terms of neuronal cells such as the DRG neuron, the mechanism of lysosomal exocytosis [35] and vesicular release of ATP have been separately reported [5–7]. However, ATP release from the DRG neurons through lysosomal exocytosis has not been clearly defined. ATP is established as a key molecule for both of an intracellular energy source and an extracellular messenger. ATP released from various cells in the nervous system plays important roles in many aspects of physiological and pathophysiological neurotransmission, including neuroprotection, neuron–glial interaction, neuropathic pain, etc. [36]. It is thus crucial to explore the functional significance of ATP released from the DRG neurons in terms of DRG–glia interaction in peripheral and central nervous systems. In this study, we showed the localization of Quin-positive vesicular ATP overlapped well with that of a lysosomal marker and a maker of the vesicular nucleotide transporter VNUT [28], in the primary cultured DRG neurons. Because most Quin-positive signals are colocalized with VNUT-positive vesicles in Fig. 2B, Quin-positive staining would be appropriate, at least in the present experiment, to represent releasable ATP stores in primary DRG cultures. These ATP containing LAMP1- and VNUT-positive vesicles are also
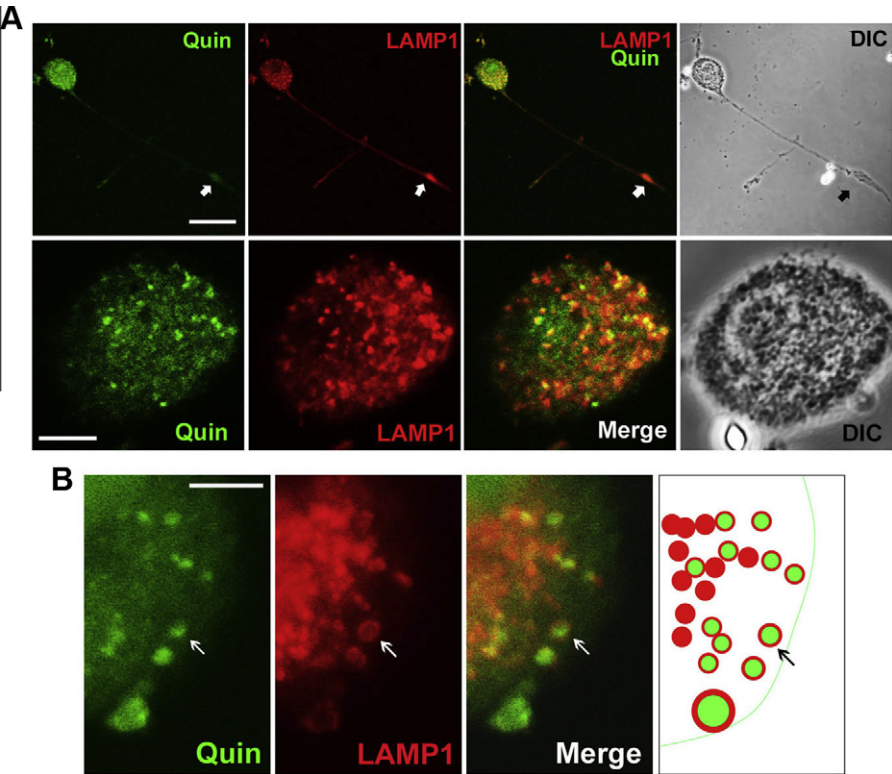
Fig. 1. ATP-containing lysosomal vesicles are present in the soma and the distal region of growing neurite of DRG neurons. (A) Top, Confocal images show the accumulation of the quinacrine (Quin) for ATP staining (arrow, green) in neuronal lysosomes labeled with anti-LAMP-1 antibody (red), and DIC of the DRG neuron. Bottom, high magnification images of DRG cell bodies. Size bar = 50 μm (top) and 15 μm (bottom). (B) High magnification images of the cell body of DRG neuron. ATP stainings are clearly surrounded by lysosomal vesicles (arrow). Size bar = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
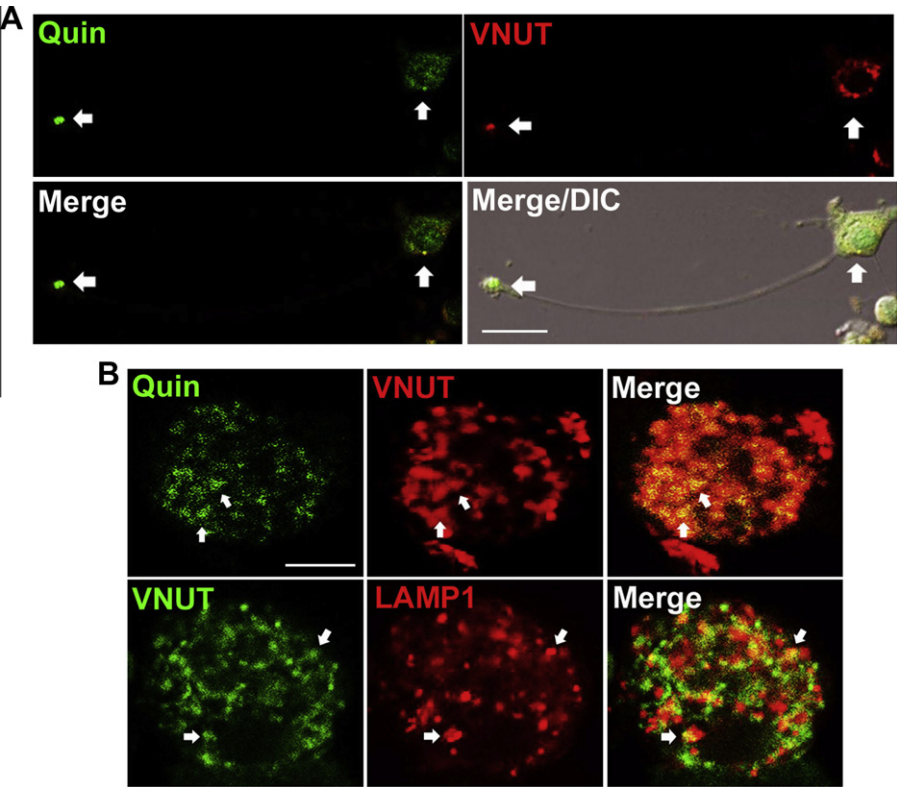

Fig. 2. A vesicular nucleotide transporter is located to ATP-containing lysosomal membrane. (A) Quin-positive vesicular nucleotide transporter (VNUT) was immunostained in neuronal soma and the tip of elongating process of cultured DRG neuron (arrows). Sized bar = 50 μm. (B) High magnification images showed that Quin-positive VNUT immunostaining (top, arrows) and VNUT-positive LAMP1 staining (bottom, arrows). Size bar = 15 μm.
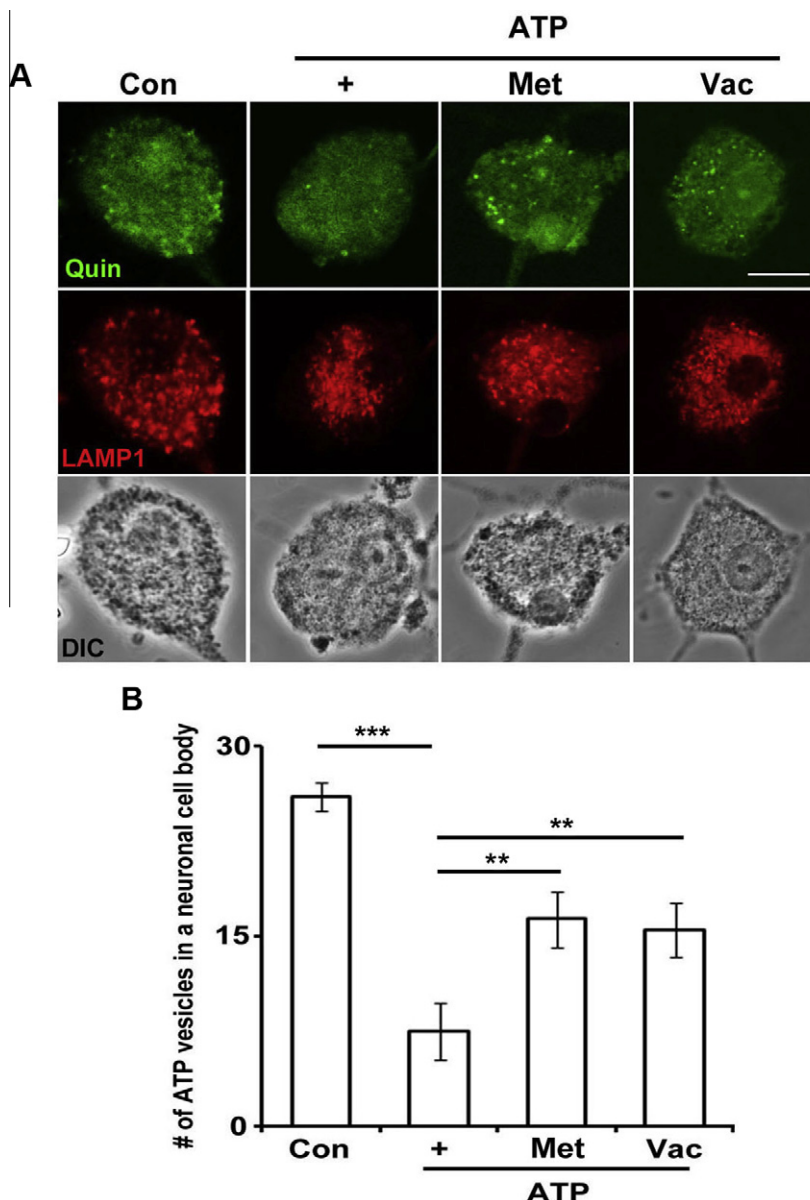
Fig. 3. Lysosomal exocytosis inhibitors inhibit ATP release from DRG neurons. (A) ATP-induced ATP release from primary DRG neurons were inhibited by lysosomal exocytosis inhibitors. Quin staining (green) colocalizes with LAMP1-positive vesicles (red). ATP (4 mM); Metformin (Met, 500 µM); Vacuolin-1 (Vac, 1 µM). Size bar = 15 µm. (B) Quantitative analysis of Quin-positive vesicles in the soma of DRG neurons. The data are expressed as the mean ± SD of average values of Quin-positive staining number from independent experiments (n = 3–5). **$P < 0.05$ and ***$P < 0.001$ compared with ATP-treated sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

present within the distal regions of neurites (Figs. 1 and 2). Thus, our data suggest that ATP could be transported into lysosomal vesicles via VNUT, and the lysosomal vesicles could be transported to the neurite tips.

Interestingly, the distribution of Quin-positive ATP staining in the DRG soma overlaps with, but not identical to, that of lysosomal vesicles (Fig. 1). We found that ATP existed predominantly in the marginal zone of the DRG soma, whereas lysosomal vesicles are evenly distributed in the cytoplasm of the DRG soma. Most of ATP in the marginal zone of DRG soma is stored inside lysosomal vesicles. It may be possible that ATP enters lysosomal vesicles through VNUT during secretory pathway of lysosomes. In addition, ATP-containing lysosomal vesicles in the tips of the neuronal process demonstrated a possibility that ATP is released from axon terminal in the dorsal horn through lysosomal exocytosis. The released ATP could induce microglial activation and neuropathic

pain [37]. Although further *in vivo* studies will be required to imply whether the lysosomal ATP-release from DRG neurons occurs in the dorsal horn of spinal cord, the present study suggests a possibility *in vitro*.

Lysosomal pH increase by chemicals, which cause alkalinization of lysosomes, results in lysosomal exocytosis [16]. In this study, we showed ATP release from DRG through lysosomal exocytosis using lysosomal exocytosis inhibitors and activators. It is reported that Met increases phagocytosis and acidifies lysosomal/endosomal compartments in rat primary microglia [30], and the triazine-based molecule, Vac inhibits Ca^{2+}-dependent lysosomal exocytosis [31]. These lysosomal exocytosis inhibitors could inhibit ATP release induced by the zymosan as a lysosoma exocytosis activator [34] in DRG neurons (Fig. 4A and B). Although the number of Quin staining could be decreased by lysosomal exocytosis, the number of lysosomal vesicles remained unchanged in the DRG soma after
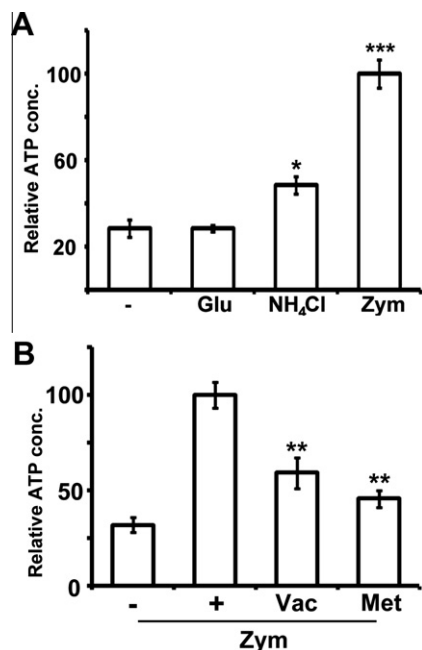
Fig. 4. ATP release is dependent on lysosomal exocytosis in DRG neurons. (A) Effect of zymosan (Zym) as a lysosomal exocytosis agonist on ATP release in DRG neurons. Zym (200 μg/mL) and NH_4Cl (4 mM) induced ATP release, while glutamate (glu, 1 mM) did not significantly increase ATP release from DRG neurons. (B) Effect of lysosomal exocytosis inhibitors on Zym-induced ATP release. Vac and Met significantly deceased the effect of Zym on lysosomal exocytosis. In figure A and B, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were compared with Zym-treated sample.

treatment with ATP (Fig. 3A and B). Previous study showed that acidification of lysosomal vesicles induced to decrease the number of lysosomes [38]. Taken together, these results suggest that ATP-induced lysosomal activation may increase not only lysosomal exocytosis but also the number of lysosomal vesicles.

In conclusion we showed a possibility that ATP could be released from the DRG neurons partly through lysosomal exocytosis after stimuli, and the regulation of lysosomal exocytosis could control ATP release from neurons. Thus, the present findings may be useful for broadening our understandings on the physiological mechanisms of ATP release from neurons and may provide a potential target for developing therapeutic treatments in nerve injury and neuropathic pain.

Acknowledgments

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