INCREASE OF TRANSCRIPTION FACTOR EB (TFEB) AND LYSOSOMES IN RAT DRG NEURONS AND THEIR TRANSPORTATION TO THE CENTRAL NERVE TERMINAL IN DORSAL HORN AFTER NERVE INJURY

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Abstract—In the spinal dorsal horn (DH), nerve injury activates microglia and induces neuropathic pain. Several studies clarified an involvement of adenosine triphosphate (ATP) in the microglial activation. However, the origin of ATP together with the release mechanism is unclear. Recent in vitro study revealed that an ATP marker, quinacrine, in lysosomes was released from neurite terminal of dorsal root ganglion (DRG) neurons to extracellular space via lysosomal exocytosis. Here, we demonstrate a possibility that the lysosomal ingredient including ATP released from DRG neurons by lysosomal-exocytosis is an additional source of the glial activation in DH after nerve injury. After rat L5 spinal nerve ligation (SNL), mRNA for transcription factor EB (TFEB), a transcription factor controlling lysosomal activation and exocytosis, was induced in the DRG. Simultaneously both lysosomal protein, LAMP1- and vesicular nuclear transporter (VNUT)-positive vesicles were increased in L5 DRG neurons and ipsilateral DH. The quinacrine staining in DH was increased and co-localized with LAMP1 immunoreactivity after nerve injury. In DH, LAMP1-positive vesicles were also co-localized with a peripheral nerve marker, Isoclectin B4 (IB4) lectin. Injection of the adenovirus encoding mCherry-LAMP1 into DRG showed that mCherry-positive lysosomes are transported to the central nerve terminal in DH. These findings suggest that activation of lysosome synthesis including ATP packaging in DRG, the central transportation of the lysosome, and subsequent its exocytosis from the central nerve terminal of DRG neurons in response to nerve injury could be a partial mechanism for activation of microglia in DH. This lysosome-mediated microglia activation mechanism may provide another clue to control nociception and pain. © 2015 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: lysosomal exocytosis, dorsal horn, spinal nerve injury, dorsal root ganglion neurons, microglia.

INTRODUCTION

Adenosine triphosphate (ATP) was established as the source of free energy involved in biochemical pathways. However, ATP is now recognized as a key molecule both of an intracellular energy source and an intercellular signaling (Fields and Stevens, 2000). It has been demonstrated that ATP is released from axons in vitro (Holton, 1959; Soeda et al., 1997; Vizi et al., 1997; Jung et al., 2013) and involved in synaptic transmission (Engelmann and Mac Dermott, 2004), and currently the role of ATP is established as a transmitter of relevant purinergic signaling (Pankratov et al., 2002; Zhang et al., 2003). ATP in the central nervous system (CNS) could be a crucial neurotransmitter because ATP-mediated activation of P2X and P2Y receptors in postsynaptic neuron, microglia, and astrocyte can trigger significant Ca2+ entry into cytoplasm and influence many metabolisms within cells (Lalo et al., 1998; Abbracchio et al., 2009). A recent study revealed that microglia express several ATP receptors after nerve injury and that released ATP contributed to the activation of microglia (Inoue et al., 2007). In addition, ATP-induced microglial activation in the spinal dorsal horn (DH) caused the neuropathic pain (Tsuda et al., 2003; Maeda et al., 2010). Thus, the regulation of ATP release in DH may be crucial for the treatment of neuropathic pain associated with microglial activation. However, the mechanisms underlying ATP release to the extracellular space in DH are poorly understood.

Proposed pathways of ATP release from cells are vesicular exocytosis and diffusion via transmembrane pores (Pankratov et al., 2006; Imura et al., 2013). Previous reports have shown that ATP was stored in presynaptic terminals and it released mainly through vesicular exocytosis (Morel and Meunier, 1981; Iijima, 1983;
Terrian et al., 1989; Sawynok et al., 1993). In our previous in vitro study, we suggested that ATP in lysosomes could be released from primary cultured dorsal root ganglion (DRG) neurons via lysosomal exocytosis (Jung et al., 2013). Lysosomes are acidified, enzyme-containing intracellular organelles to break down phagocytosed materials, cell debris and waste materials (Holtzman, 1989). Recently, it was identified that lysosomes have an additional property for regulatory exocytosis (secretory lysosomes) (Blott and Griffiths, 2002). The exocytotic process of mature lysosomes can be triggered by an increase of intracellular free Ca$^{2+}$ (Andrews, 2000; Blott and Griffiths, 2002). Then, a microtubule-dependent step provides the movement of exocytic lysosomes toward the plasma membrane (Andrews, 2000). The lysosomal exocytosis also can be triggered by chemicals that cause alkalization of lysosomes (Sundler, 1997). The lysosomal materials such as enzymes, degraded molecules, ions and ATP, which are all released by lysosomal exocytosis, could be mediators for the activation of microglia.

In the present study, we addressed a possibility that lysosome is exocytosed from central nerve terminal of DRG neurons into DH after peripheral nerve injury (Kim and Chung, 1992; Tsuda et al., 2003). We here demonstrated that peripheral nerve injury induced the transcription factor EB (TFEB) mRNA expression in DRG and subsequent increase of vesicular-nuclear-transporter (VNUT)-positive lysosomal vesicles in both DRG neurons and DH suggesting up-regulation of lysosomal synthesis and ATP packaging in DRG neurons. In addition the transportation of lysosomes from DRG to DH was also confirmed by injecting adenovirus encoding fluorescence-labeled lysosomal protein. The results suggested that lysosomal exocytosis in DH after peripheral nerve injury may be partly associated with microglial activation in DH.

**EXPERIMENTAL PROCEDURES**

**Materials**

The primary antibodies against LAMP1 (Sana Cruz Biotechnology, Sana Cruz, CA, USA, Cat# sc8098, RRID: AB_2134494; Cat# sc71489, RRID: AB_2265605) were used for immunostaining or Western blotting. Phospho-p44/42 MAPK (p-ERK1/2, Cat# 9101S, RRID: AB 331046) and p44/42 MAPK (ERK1/2, Cat# 9102S, RRID: AB 10695746) were obtained from Cell signaling (Beverly, USA). VNUT and ionized calcium binding adapter molecule 1 (Iba-1) were obtained from MBL Co., Ltd (Woburn, MA, USA, Cat# BMP079, RRID: AB 10597575) and Wako (Osaka, Japan, Cat# 019-19741, RRID: AB 839504), respectively. IB4 and all other reagents were purchased from Sigma–Aldrich (St. Luis, MO, USA).

**Animals and surgical procedure for nerve injury**

All animal experiments have been carried out in accordance with EU Directive 2010/63/EU for animal experiments. Also all animal experiments have been carried out in accordance with the University Animal Committee “Guidelines for the Care and Use of Laboratory Animals”, and were approved by the Nagoya University Institutional Animal Care and Use Committee. All possible efforts were made to minimize suffering. Adult male Wistar rats (7 weeks rats) were obtained from SLC (Hamamatsu, Japan). Animals were housed with a 12-h light/dark cycle (8:00/20:00) at a constant room temperature (RT) of 23 ± 2 °C and humidity of 45–65%. After induction of anesthesia by intraperitoneal (i.p.) injection of pentobarbital (40 mg/kg), the L5 nerve was tightly ligated with a 5-0 silk and cut distal to the ligature as previously described by (Tozaki-Saitoh et al., 2008).

**Tissue processing**

Seven days after L5 spinal nerve ligation (SNL), the L5 spinal cord were collected and stored as appropriate for each experiment. For immunohistochemistry (IHC), tissues were perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) and then excised. The samples were post-fixed by the same solution overnight at 4 °C and treated by with 30% sucrose in 0.1-M PB for 3 days. They were then embedded in OCT and frozen immediately on dry ice. Frozen sections (16 μm) were cut from tissues and put on the polylysine-coated slide glasses (Fisher Scientific, Houston, TX, USA).

For the analysis of protein and RNA, the spinal cord was isolated from animals immediately after sacrifice. The spinal cord was separated into DHs at the L5 segment. Each sample was homogenized in a modified radioimmunoprecipitation assay buffer (RIPA; 50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium o-vanadate, and protease inhibitor cocktail [Roche Molecular Biochemicals, Nutley, NJ, USA]). Protein concentrations were quantified by a Bradford assay following standard protocols. Total RNA was extracted from each sample by the conventional acid guanidine isothiocyanate/phenol/chloroform extraction (AGPC) method (Chomczynski and Sacchi, 2006).

**Immunohistochemistry**

Before staining, sample slides were fixed in 4% PFA for 10 min. Following three washes in phosphate-buffered saline (PBS), the samples were permeabilized in PBS containing 0.3% Triton X-100 (PBST) and blocked with 5% bovine serum albumin (BSA) and 5% fetal bovine serum overnight at 4 °C. Primary antibodies for each molecule were placed on the slides, which were incubated for 16 h at 4 °C. Following three washes in PBS, slides were incubated for 2 h at RT with Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA), Alexa Fluor 488 (Invitrogen, USA) or Alex Fluor 647 (Invitrogen, USA) secondary antibodies. The slides were then washed three times with PBS, slide-mounted and subsequently cover-slipped. The immunolabeling was analyzed using an LSM700 imaging system (Carl Zeiss, Oberkochen, Germany).

**Western blotting**

Western blotting analysis was performed with the tissues described above. Protein extracts were separated using 10% sodium dodecyl sulfate polyacrylamide gel
electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare, Waukesha, WI, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20.

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Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed with the tissues described above. In brief, isolated RNA was obtained from L4–L6 spinal cord and then reverse-transcribed with random 6-mer oligo dT using SuperScript II (Invitrogen Corporation, USA). RT-PCR was performed using the specific primers as follows: TFEB, forward, 5’-GGTGCACTCCTGGAGAGA-3’; reverse, 5’-CCGCTCTGTCGCTTCCCTTG-3’; Neu-1, forward, 5’-TCGGCTGATAGAAGACCTTT-3’; reverse, 5’-GTGTCATCAGTGGAGGAGA-3’, VNTT, forward, 5’-GCTCTACGTGGTCATCAGG-3’, GAPDH, forward, 5’-GATGTGCTCCT-3’; reverse, 5’-GCCTGGAGAGA-3’, LAMP1, forward, 5’-GCTGAGTGGACTGTGGTCATG-3’, reverse, 5’-GCTGAGTGGACTGTGGTCATG-3’, LAMP1, forward, 5’-GCTGAGTGGACTGTGGTCATG-3’, reverse, 5’-GCTGAGTGGACTGTGGTCATG-3’. RT-PCR was performed using 30 PCR cycles depending on the target gene, with annealing temperatures of 60°C. PCR products were visualized by 1% agarose gel electrophoresis followed by GelRed (Biotium, Hayward, CA, USA) staining.

ATP staining

For ATP staining in vivo, we used quinacrine dihydrochloride as a fluorescent dye. Quinacrine staining was performed as described in previous report (Lee et al., 2013). Briefly, ATP staining was performed by i.p. injection with quinacrine (50 mg/kg) in PBS to rats three times per day (Belai and Burnstock, 1994). Slide preparation was performed as described above.

Adenovirus production and its delivery in vivo

Cosmid pAxCALNLmLAMP1-mCherry and pAxCaWCre were constructed by the insertion of mouse LAMP1-mCherry or Cre recombinase sequence into the SwaI cloning site of pAxCALNLw or pAxCAwt respectively. Using a Cell Phect transfection kit (GE Healthcare, Cat# 27-9268-01), recombinant adenovirus of AxCALNLmLAMP1-mCherry and AxCaWCre was generated by transfecting 293 cells with DNA-TPC (Takara Shuzo, Japan) and pAxCALNLmLAMP1-mCherry or pAxCaWCre. When these two viral vectors co-infected cells, Cre recombinase expressed under the CAG promoter activates the stuffer PolyA through the Cre/LoxP system. Recombinant adenovirus particle titers (VP) were determined spectrophotometrically. Each adenoviral vector was used at the concentration of 1.5 × 10^9 VP/µL (AxCALNLmLAMP1-mCherry) or 5.0 × 10^8 VP/µL (AxCaWCre). The viral solutions were injected into L5 DRG (total, 1.0 µL) concurrently when L5 nerve was injured.

Statistical analysis

Differences between groups were statistically analyzed using an analysis of variance followed by Bonferroni’s post hoc test. Data were assessed as mean ± SEM. Values were considered to be statistically significant at p < 0.01 after the analysis of four independent experiments.

RESULT

Increase of LAMP1 expression and TFEB mRNA in DRG neurons after nerve injury

To confirm whether the nerve injury increases lysosomal vesicles in DRG neurons in vivo as shown in the previous in vitro result (Jung et al., 2013), we immunostained injured L5 DRG with anti-LAMP1 antibody. Activating transcription factor 3 (ATF3) was used for a neuron-injury marker (Takeda et al., 2000; Tsujino et al., 2000; Ohba et al., 2003). A significant increase of LAMP1 immunoreactivity was observed in the ATF3-positive DRG neurons (Fig. 1A–C). Next, we examined a possibility that the ATP stores are associated with lysosomal vesicles in a DRG neuron using quinacrine staining and immunohistochemistry with anti-LAMP1 antibody. Fig. 1D shows clear merged signals of LAMP1 and quinacrine in a DRG neuron cell body. These data indicate that ATP could be localized in lysosomal vesicles in DRG neurons in vivo as well as DRG in vitro (Jung et al., 2013). The increase of LAMP1 mRNA in in vivo DRG neurons was validated independently by semi-quantitative RT-PCR analysis.

Recent studies have reported that TFEB activates lysosomal exocytosis (Medina et al., 2011; Settembre et al., 2011), but neuramidase-1 (Neu-1) negatively regulates lysosomal exocytosis (Yogalingam et al., 2008). To confirm induction of lysosomal synthesis and exocytosis in injured DRG neurons, we performed RT-PCR analysis with specific primers for LAMP1, TFEB and Neu-1 mRNAs. We found that LAMP1 and TFEB mRNA levels were significantly increased in the ipsilateral DRG neurons compared with contralateral DRG neurons, but no alteration of Neu-1 mRNA expression level in the injured DRG (Fig. 2A, B). These results indicate that mRNAs for TFEB and LAMP1 as well as the number of lysosomes are concomitantly increased in the injured DRG neurons, suggesting that induced TFEB expression activates lysosomal synthesis and possibly lysosomal exocytosis in DRG neurons after nerve injury.

Increase of VNTT expression in DRG neurons after nerve injury and co-localization with LAMP1

Previous studies have revealed that VNTT plays a crucial role in transporting ATP into vesicles in the several cell types (Shehab et al., 2004; Sawada et al., 2008; Jung...
et al., 2013). We therefore immunostained rat DRG sections with anti-VNUT and anti-LAMP1 antibodies. The VNUT immunoreactivity was significantly increased in the injured side compared with uninjured side of L5 DRG (Fig. 3A, B). The increased VNUT immunoreactivities were also overlapped with LAMP1-positive profiles in the injured side (Fig. 3C). RT-PCR analysis also indicated the increased mRNA expression level of VNUT in the injured DRG neurons compared with the control DRG neurons (Fig. 3D, E). Thus, these findings suggest lysosomal vesicles are capable of storing ATP through VNUT in DRG neurons.

Increase of LAMP1 immunoreactivity in the ipsilateral DH after nerve injury

To determine the alteration of lysosomal localization in the spinal DH after sciatic nerve injury, we performed
immunostaining using sections from the L5 level of spinal cord with anti-LAMP1 antibody. We found that LAMP1 immunoreactivity was significantly increased in the ipsilateral side of spinal DH (Fig. 4A). Previous studies have reported that spinal nerve injury induces microglial activation in the injured side of spinal DH (Watkins and Maier, 2003; Tsuda et al., 2005). We confirmed the microglial activation and increase in the lysosome-increased ipsilateral DH using Iba-1 antibody (Fig. 4A).

In addition, the increase of LAMP1 immunoreactivity was confirmed by Western blot analysis. LAMP1 expression in DH at day 1 after nerve injury was significantly increased and the increase was maintained at day 7 after nerve injury (Fig. 4B, C). The increase of
LAMP1 in DH after nerve injury had continued for 3 weeks (data not shown). A previous study showed that pERK1/2 activation occurs in the spinal DH after nerve injury (Zhang et al., 2007b). We used pERK1/2 as a positive control and ERK1/2 as a loading control. To assess whether the increase of LAMP1 immunoreactivity is in microglia or not, we performed simultaneous visualization of LAMP1 and Iba-1 immunoreactivities. Many large LAMP1-positive puncta were observed in ipsilateral DH. Many of LAMP1 immunoreactivities did not co-localize with Iba-1 immunostaining in the ipsilateral side of DH (Fig. 4D). These results indicate that the increased large lysosomes mainly exist out of microglia after SNL.

Next, we assessed whether the ATP stores are associated with lysosomal vesicles in DH using quinacrine staining and immunohistochemistry with anti-LAMP1 antibody. On day 6 after SNL, rats were injected with quinacrine and, the next day, spinal cords were sampled for analyses. In L5 spinal cord sections, quinacrine staining was apparently more detectable in the ipsilateral DH compared to the contralateral side of the DH after SNL (Fig. 5A). In addition, the most prominent quinacrine-positive staining was found in the laminae I and II of the ipsilateral DH (Fig. 5A arrows). Low basal constitutive quinacrine staining was found in the contralateral region of DH as well as in cell bodies of motor neurons in the ventral horn (Fig. 5A).

In superficial layers of DH, the quinacrine staining was co-labeled with LAMP1 (Fig. 5B). High-magnification images also showed that the most of intense quinacrine staining co-localized with LAMP1 (the lowest panel in Fig. 5B). Although all LAMP1 immunoreactivities were not co-localized with the quinacrine staining, most of quinacrine staining showed co-localization with LAMP1.
immunoreactivity, suggesting that there exist various types of lysosomes with abundant to less ATP. A quantitative analysis indicated that LAMP1-positive signals (Fig. 5C) and quinacrine-positive signals (Fig. 5D) were significantly increased in the ipsilateral DH compared with the contralateral side after SNL. Percentage of quinacrine/LAMP1 double-positive signals in the total of LAMP1-positive signals indicated no significant difference between the ipsilateral and contralateral side of DH after SNL (Fig. 5E). Taken together, these results suggested that the number of ATP containing vesicles is increased in the injured side of DH after nerve injury and the increased ATP storage would be lysosomal vesicles in DH.

Increase of VNUT immunoreactivity in the injured DH and co-localization with LAMP1 immunoreactivity

Since a higher rate of co-localization of VNUT and LAMP1 immunoreactivities was observed in DRG neurons after nerve injury, we also examined their co-localization in DH. The increases of mRNAs for LAMP1 and VNUT in the ipsilateral side of DH were identified 7 days after nerve injury by RT-PCR (Fig. 6A, B). Next, we immunostained both VNUT and LAMP1 in the spinal cord sections. The VNUT immunostaining was significantly increased in the ipsilateral side compared with contralateral side of spinal DH (Fig. 6C), and VNUT immunoreactivities were co-localized with LAMP1-positive profiles significantly in the ipsilateral side compared with the contralateral side of DH (Fig. 6D, E). These findings suggest that lysosomal vesicles in DH are capable of storing ATP through VNUT.

Co-localizations of quinacrine/IB4 in the nociceptive central afferent terminals in vivo

We previously reported that lysosomal activation is increased in the process terminal of primary cultured DRG neurons (Jung et al., 2013). We therefore examined whether the LAMP1-positive vesicles locate in the central terminals from DRG neurons. To examine a possibility that the increased LAMP1 immunoreactivity seen in DH after nerve injury is in the central branch of DRG neurons,
we compared LAMP1 immunoreactivity with IB4. We used IB4 as a maker of C-fiber nociceptor central terminals. Our IB4 staining existed in the afferent central terminal of lamina II (Munglani et al., 1995; Shehab et al., 2004) and the most prominent LAMP1 immunoreactivity was found in the laminae II of the ipsilateral DH within IB4 binding region (Fig. 7A, B). Next, we compared quinacrine staining with IB4 binding. We found that quinacrine staining overlapped with IB4 staining in the ipsilateral DH (Fig. 7C). Quantitative analysis showed that quinacrine/axon marker IB4 double-positive signals were increased in the ipsilateral side of L5 spinal DH (Fig. 7D). Thus, these findings indicate that the increase of quinacrine staining and lysosomal vesicles may occur in the axon terminals originated from the injured DRG neurons.

**Localization of DRG-originated LAMP1 in DH**

To evaluate the transportation of lysosomal vesicles to DH from DRG neurons more directly, we constructed adenovirus vector expressing LAMP1-mCherry. 7 days after infecting L5 DRG neurons with the LAMP1-mCherry-expressing adenovirus, a few L5 DRG neurons expressed the mCherry fluorescence (Fig. 8A), suggesting a minor DRG neurons were infected and expressed the mCherry-LAMP1. In the ipsilateral DH, mCherry was co-localized with IB4 (Fig. 9A–C arrows), but not with Iba1 (a marker for microglia, Fig. 9D–F). This indicated that mCherry-labeled lysosomes

![Fig. 6. Increases of VNUT and LAMP1 mRNAs and their immunoreactivities in the injured DH. (A) RT-PCR analysis of LAMP1 and VNUT mRNAs in the spinal dorsal horn on day 7 after L5 SNL. GAPDH is a loading control. Cont, contralateral; Ipsi, ipsilateral. (B) Quantification of the relative intensity of bands derived by RT-PCR. **p < 0.01 (n = 3). (C) Confocal images showing that the vesicular nucleotide transporter (VNUT, green) immunoreactivity was increased in the ipsilateral side compared with contralateral side of DH after L5 SNL. Scale bar = 200 μm. (D) High magnification images of VNUT (green) and LAMP1 (red) immunostaining in DH. Arrows indicate co-localization of VNUT and LAMP1. Scale bar = 20 μm. (E) Quantitative analysis showed that nerve injury induced VNUT-immunoreactivity profiles co-labeled with LAMP1-immunoreactivity per 100 μm² in the ipsilateral DH after nerve injury. *p < 0.01 (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
synthesized in IB4-positive DRG neurons were transported to DH. Obviously the mCherry-labeled lysosomes were considered as DRG-originated lysosomes. Collectively, these findings support an idea that the increase of quinacrine staining and lysosomal vesicles in DH after nerve injury would be partly due to the transportation of ATP containing lysosomes from DRG neurons.

**DISCUSSION**

In this study, we demonstrated that nerve injury-activated lysosomal biosynthesis in DRG neurons and VNUT-positive lysosomes were transported to the primary afferent nerve terminal in DH (Fig. 10). We found that the injury of peripheral process of DRG neurons induced the expression of TFEB mRNA (Fig. 2A, B). TFEB is a master transcriptional regulator of lysosomal biogenesis, and the TFEB transcriptionally induces both the docking and fusion of lysosomes with the plasma membrane by regulating the expression of certain genes (Medina et al., 2011; Settembre et al., 2011, 2013). This suggests that the increase of lysosomal vesicles in DRG neurons could be due to the induction of TFEB in DRG neurons. Intriguingly, together with the increase of lysosomes in DRG neurons, VNUT mRNA was induced in the same DRG neurons, and prominent co-localization of VNUT and LAMP1 was observed on vesicles. This would suggest that ATP storage in lysosomes is activated in nerve-injured neurons for some reason.

What are the functional consequences of the lysosome increase in injured DRG neurons? One most likely explanation would be an involvement of lysosomes in membrane repair at injured peripheral nerve process (Settembre et al., 2013; Appelqvist et al., 2013). Previous
studies have reported that lysosomal vesicles are involved in the process of membrane repair through Ca$^{2+}$-dependent synaptotagmin VII in the damaged cell by a way to fuse with the plasma membrane (Gerasimenko et al., 2001; McNeil, 2002; Idone et al., 2008; Settembre et al., 2013; Appelqvist et al., 2013). The present nerve injury might induce the influx of Ca$^{2+}$ into DRG neurons and subsequently induces the number of lysosomal vesicles and lysosomal exocytosis under the activation of TFEB. The increased fusion of lysosomes with plasma membrane could function to repair the injured neurite of DRG neurons. The second possibility would be an intracellular clearance of molecules and organelles by lysosomal activation and exocytosis. Under pathological conditions, TFEB-mediated activation of lysosomal biogenesis and exocytosis would be advantageous for removing intracellular toxic materials and injured organelle to outside of cell membrane (Medina et al., 2011; Settembre et al., 2013). Nerve injury elicits various types of stresses including oxidative stress and ER stress in neurons and these stresses would damage organelle and induce accumulation of unfolded proteins. For the removal of those unnecessary or even toxic contents, lysosomal degradation and clearance including autophagy would be necessary in nerve-injured neurons. Although both the lysosome-mediated membrane repair and intracellular clearance are critical in injured peripheral process and soma of DRG neurons, it is likely that the increased lysosome plays some role in the central process as well. DRG neurons have both peripheral and central processes and it appears that no directional preferences to be transported exist for some neurotransmitters such as substance P and organelle such as mitochondria at least (Harmar and Keen, 1982). In fact, the present adenovirus-mediated expression of mCherry-LAMP1 clearly demonstrated the transport of lysosomes in the central branch of DRG neurons. Thus, in response to nerve injury, ATP-containing lysosomes would be delivered to both central and peripheral terminals of DRG neurons and whereby the ingredients including ATP might be released.

Recently lysosomes are considered as one of storage vesicles for ATP in neuronal and glial cells, and the lysosomal exocytosis possibly releases ATP together with other ingredients into the extracellular space (Zhang et al., 2007a; Pryazhnikov and Khiroug, 2008; Shin et al., 2012; Jung et al., 2013). The present co-localization study of LAMP1 and quinacrine in the same vesicle suggested that the increased lysosomal vesicles in the ipsilateral DH after nerve injury also contain ATP (Fig. 5). Because the existence of ATP in lysosomes and ATP release by lysosomal exocytosis were demonstrated in cultured DRG neurons (Jung et al., 2013), it is likely that the lysosomal exocytosis is a mechanism underlying ATP release in DH after nerve injury. Substantial increase of lysosomal protein, LAMP1, was demonstrated in the injured DH by immunohistochemistry and Western blot analysis for LAMP1 (Figs. 4 and 5). Thus, these findings suggest that the lysosomal ATP may be associated with nociceptive pathophysiology because the activation of ATP containing lysosomes occurred in the area where the central terminals of nociceptive c-fiber from DRG exist (Figs. 7–9).

Several types of axons and processes including the central process of DRG neurons locate in DH. Because quinacrine- and LAMP1-positive vesicles were highly increased in DRG neurons after nerve injury as shown in Figs. 1–3 and our previous study (Jung et al., 2013), it is very likely that ATP containing lysosomes is transported to DH in response to peripheral nerve injury. To clarify this possibility, peripheral nerve marker IB4 was used and LAMP1-positive structures existed jointly with IB4 immunoreactivity in the ipsilateral DH with adenoviral infection (Figs. 8 and 9). In addition, we showed the increase of VNUT expression in the ipsilateral DH and the injured DRG neurons and its expression in lysosomal vesicles in vivo (Figs. 3 and 6) as shown in our previous...
in vitro study (Jung et al., 2013). These findings together with our previous study (Jung et al., 2013) would support our hypothesis that peripheral nerve injury induces the transportation of ATP-containing lysosomes to the central branch of DRG neurons and suggest a possibility that ATP is released from axon terminals of DRG neurons through lysosomal exocytosis in addition to releases from astrocytes and microglia (Zhang et al., 2007b; Dou et al., 2012).

**CONCLUSION**

We demonstrated the transcriptional activation of lysosomal exocytosis activator TFEB in DRG neurons in response to nerve injury and prominent increase of lysosomes was also observed in both DRG and DH of the spinal cord where DRG neurons project. Since it becomes evident that the transportation of lysosomes from DRG to DH through the central branch of primary
afferent neurons, it is likely that ATP in addition to some additional ingredients are released by the lysosomal exocytosis at the central terminal of DRG neurons. Although we could not demonstrate direct release of ATP from the primary afferent nerve terminal in DH via lysosomal exocytosis, previous in vitro study supported the possibility (Jung et al., 2013). Thus, a regulation of lysosomal exocytosis at the axon terminals of DRG neurons may provide an alternative clue to control nociception and pain.

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