Autophagy in superficial spinal dorsal horn accelerates the cathepsin B-dependent morphine antinociceptive tolerance

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Abbreviations:

ANOVA: analysis of variance

CatB, cathepsin B

CA074 Me, [L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline
Methyl Ester

EPSC, excitatory postsynaptic current

GAD: glutamic acid decarboxylase

LC3:(MAP1LC3), Microtubule-associated protein light chain 3

3MA, 3-methyladenin

Abstract

Opioids are the most widely used analgesics in the treatment of severe acute and chronic pain. However, opioids have many adverse side effects, including the development of antinociceptive tolerance after long-term use. The antinociceptive tolerance of opioids has limited their clinical use. A recent study has reported that autophagy is responsible for morphine-induced neuronal injury. However, little is known about the role of autophagy in morphine antinociceptive tolerance. In the present study, chronic morphine administration was found to induce the expression of autophagy-related proteins, including Beclin1 and microtubule-associated protein light chain 3 (LC3)-II, in GABAergic interneurons in the superficial layer (lamina I-II) of the spinal cord. A single intrathecal administration of autophagy inhibitors, 3-methyladenine (3MA) or wortmannin, inhibited the development of antinociceptive tolerance in a dose-dependent manner. Autophagy in the lamina I-II neurons was associated with increased level of cathepsin B (CatB), a lysosomal cysteine protease. The pharmacological blockade or gene deletion of CatB markedly prevented the development of morphine antinociceptive tolerance. Furthermore, the intrathecal administration of 3MA suppressed the upregulation of CatB 5 days after morphine administration. Finally, CatB deficiency inhibited the increased release probability of glutamate in the lamina I neurons after chronic morphine treatment. These observations suggest that the dysfunction of spinal GABAergic system induced by CatB-dependent excessive autophagy is partly responsible for morphine antinociceptive tolerance following chronic treatment.

Keywords: morphine, antinociceptive tolerance, autophagy, Cathepsin B, GABAergic interneuron, disinhibition, microglia

1. Introduction

The opioids are the most effective medicine for severe pain, including cancer pain and post-operative pain. However, opioids have many adverse side effects, including respiratory depression, nausea, sedation, euphoria or dysphoria, decreased gastrointestinal motility and itching (Inturrisi, 2002). Long-term administration of opioids gradually decreases their analgesic effects at the equivalent dose. Therefore, dose escalation is required for effective pain relief. Better understanding of the mechanisms underlying the antinociceptive tolerance of opioids is needed to improve the treatment of millions of patients suffering from chronic pain syndromes.

Several mechanisms have been proposed to underlie the antinociceptive tolerance of opioids. For instance, chronic opioid treatment activates protein kinase C and *N*-methyl-D-aspartic acid receptor signaling and induces the desensitization of mu opioid receptors through the beta-arrestin 2 in the spinal cord (Bohn et al., 2000). Tonic activation of descending pain facilitation system arising in rostral ventromedial medulla is another causative factor for the development of morphine antinociceptive tolerance (Vanderah et al., 2001). Recently, a unique phenomenon of morphine was discovered that chronic activation of mu opioid receptor initiated autophagy in hippocampal neurons (Zhao et al., 2010). However, the role of autophagy in morphine antinociceptive tolerance remains unclear.

Autophagy is a reparative, life-sustaining process in which cytoplasmic components are sequestered in double-membrane vesicles and degraded upon fusion with lysosomal compartments. The contents of the lysosomes eventually are degraded into amino acids to maintain cellular energy (Kabeya et al., 2000). Thus, autophagy plays an essential

quality-control function in the cell by promoting basal turnover of long-lived proteins and organelles, as well as by selectively degrading damaged cellular components. On the other hand, excessive autophagy leads to self-destruction, culminating into cell death. The accumulation of abnormal autophagosomes is one of the pathological hallmarks of several neurological disorders, including Alzheimer's disease, Huntington's disease and Parkinson's disease (Mizushima, 2007). Therefore, a fine balance of autophagy is necessary for the normal brain function.

The aim of this study was to investigate 1) whether the autophagic process is involved in morphine antinociceptive tolerance, 2) whether autophagy has an impact on neuronal functions, and 3) whether CatB mediated signaling has key role in autophagic process following morphine administration.

2. Experimental procedures

2.1. Animals and drug administrations

The experimental protocol was approved by the Animal Research Committee of Kyushu University. All efforts were made to minimize animal suffering and to reduce the number of animals used. The adult male cathepsin B-deficient (CatB^{-/-}) (Sun et al., 2012) and wild-type littermates, on C57B/6 background (10 weeks), were used for the experiments. In some experiments, we used glutamate decarboxylase 67-green fluorescence protein (GAD67-GFP) knock-in mouse (Tamamaki et al., 2003). The mice were maintained on a 12 h light/dark cycle (light on at 8:00 AM) under conditions of 22–25°C ambient temperature with food and water ad libitum. All mice were handled daily for 5 days before the start of the experiment to minimize their stress reactions to

manipulation. Saline (0.2 mL) or morphine (10 mg/kg) was intraperitoneally (i.p.) administered twice a day (9:00 AM and 9:00 PM). In some experiments, 3-methyladenine (3MA, an autophagy inhibitor), wortmannin (an autophagy inhibitor), or CA074Me (a CatB inhibitor) was intrathecally (i.t.) injected.

2.2. Chemicals and Intrathecal Injection

Under 2% isoflurane anesthesia, 3MA (1, 3, 10, or 30 μ g, Sigma-Aldrich Co. LLC, St Louis, MO, USA), wortmannin (0.1, 0.3, or 1 μ g, Sigma-Aldrich Co. LLC, St Louis, MO, USA), CA074Me (0.1, 0.5, or 1 μ g, Merck & Co., Inc., Whitehouse Station, NJ, USA), or saline in a volume of 10 μ L was intrathecally injected with a 30-gauge needle connected to a 25 μ L Hamilton syringe as previously described (Hayashi et al., 2011). The injection was delivered 1 h prior to the morphine administration on day 1.

2.3. Behavioral analyses

The antinociceptive effects were assessed by the hot-plate (55 $\pm$ 0.5 $^{\circ}$ C) test (Muromachi Kikai, Co., Ltd., Tokyo, Japan). The latency to a respond to the heat stimulus was measured by the amount of time it took for the animal to lick one of its paws or jump. A cut-off time of 30 s was chosen to minimize the thermal damage to the mice. The hot-plate test was conducted 1 h after morphine administration.

2.4. Immunohistochemical analyses

Five days after the morphine administration, the mice were anesthetized with somnopentyl (50 mg/kg) and then perfused transcardially with 0.1 M phosphate buffer saline (PBS), pH 7.4, followed by 4% paraformaldehyde (PFA). The perfused L4 spinal cord segments were dissected and further fixed by immersion in 4% PFA overnight at 4°C. Transverse L4 spinal sections (free-floating; 50 µm thick) were prepared by a vibratome (VT1000S; Leica Microsystems GmbH, Wetzlar, Germany). The floating sections were stained with antibodies for anti-NeuN (1:5,000; EMD Millipore Corporation, Billerica, MA, USA), anti-LC3 (1:1,000; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), anti-Beclin1 (1:1,000; BD Biosciences, San Jose, CA, USA), anti-CatB (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-vesicular glutamate transporter 3 (vGluT3, 1: 2500; FRONTIER INSTITUTE Co.Ltd, Hokkaido, Japan), anti-ionized calcium-binding adaptor molecule 1 (Iba1, 1:10,000; Wako Pure Chemical Industries, Ltd, Japan), anti-glial fibrillary acidic protein (GFAP, 1:5,000; Sigma-Aldrich Co. LLC. St. Louis, MO, USA) for 5 days at 4°C using a similar method as previously reported (Hayashi et al., 2011). The immunohistochemistry for GAD67 was conducted using Triton-X free solution, because permeabilization procedure deficits GAD67 signal in the cell body. First antibody was used anti-GAD67 antibody (1: 250; Millipore). The spinal sections were visualized with the mixture of secondary antibodies conjugated with Cy3 or Alexa 488 (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 3 h at 4°C. The sections were mounted in the anti-fading medium Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). The images were captured with the C2si Confocal Laser Microscope (Nikon Corporation, Tokyo, Japan). Images were captured by ×20 (NA: 0.75) dry lens and ×60 and ×100 (NA: 1.4) oil immersion lens. To measure the

immunofluorescence (IF) intensity of Beclin1, LC3, and CatB of spinal cord, superficial layer of spinal dorsal horn was identified by vGluT3 immunoreactivity that represented the border between lamina II and III (Seal et al., 2009). ROI (region of interest) of superficial layer was determined according to the vGluT3 immunoreactivity. All the images were binarized and then measured mean gray value by ImageJ 1.47h software (National Institutes of Health, Bethesda, MD, USA). All IF intensities of the images were calibrated with average value of saline-treated group. The number of NeuN-positive cells and GAD67-GFP-positive cells in the superficial layer of spinal cord was counted and calibrated with the ROI area.

2.5. Immunoblot analyses

Each animal (0, 1, 3, and 5 days after morphine administration) was anesthetized with somnopenyl and then perfused transcardially with 0.1 M PBS. The spinal segment (lumbar 4-5) was removed, and the dorsal horn was separated. The soluble fractions that were obtained from spinal dorsal horn homogenates by differential centrifugation were electrophoresed in 15% SDS-polyacrylamide gels. The proteins on SDS gels were transferred electrophoretically to nitrocellulose membranes. The membranes were washed with PBS and incubated at 4°C overnight under gentle agitation with anti-LC3 (1:1,000; Medical & Biological Laboratories), anti-Beclin1 (1:1,000; BD Biosciences), anti-CatB (1:1,000; Santa Cruz), and anti- β -actin (1:1000; Santa Cruz). After washing, the membranes were incubated with horseradish peroxidase (HRP)-labeled anti-goat (1:1,000; R&D Systems, Inc., Minneapolis, MN, USA) or anti-rabbit (1:1000; GE Healthcare, Buckinghamshire, UK) antibodies for 1 h at room temperature.

Subsequently, the membrane-bound HRP-labeled antibodies were detected with an enhanced chemiluminescence detection system (ECL lit; GE Healthcare) with an image analyzer (LAS-1000; FujiFilm Corporation, Tokyo, Japan).

2.6. Electrophysiology

The mice (2–3 weeks old) that were subjected to morphine (i.p.) for 5 days were anesthetized with somnopentyl. The slices (200 μ m thick) were cut from the spinal cord (L4) with a vibratome as previously reported (Hayashi et al., 2011). The artificial cerebrospinal fluid contained (in mM): 117 NaCl, 3.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 11 glucose saturated with 95% O₂ and 5% CO₂. The recordings were made from lamina I neurons in the spinal cord (5–8 cells from 3 slices from 3 animals). Patch pipettes (5–10 M Ω) were filled with an internal solution containing the following components (in mM): 135 potassium gluconate, 5 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 5 HEPES and 5 ATP-Mg. All recordings were made at a holding potential of -70 mV. Miniature excitatory postsynaptic currents (EPSCs) were recorded in the presence of 1 μ M tetrodotoxin, 10 μ M bicuculline methiodide, a competitive GABA_A receptor antagonist, and 1 μ M strychnine, a competitive glycine receptor antagonist. The data were stored and analyzed according to the methods described in a previous report (Hayashi et al., 2013).

2.7. Statistical analyses

All data are shown as the mean $\pm$ s.e.m. The statistical analyses were performed

with a one-way analysis of variance (ANOVA) with a post-hoc Tukey's test or a two-way ANOVA with repeated measurements followed by Bonferroni post-hoc tests using the GraphPad Prism Software package (GraphPad Software, Inc., La Jolla, CA, USA). Unless otherwise indicated, the data met the assumptions of equal variances. Differences were considered significant with P values less than 0.05.

3. Results

3.1. A possible involvement of autophagy in the development of morphine tolerance

To assess the involvement of autophagy in the development of morphine antinociceptive tolerance, we first examined effects of 3MA and wortmannin, autophagy inhibitors, on the pain behavior of mice following treatment with morphine. Morphine (10 mg/kg, i.p. n=5) was administered twice a day. Five days after the morphine administration, the hot-plate latency returned to the baseline level (saline: 8.1 ± 1.1 s, morphine: 8.8 ± 0.5 s; Fig.1A), indicating that antinociceptive tolerance was fully established at day 5. A single injection of 3MA (30 μ g/10 μ L, i.t., n=12) on day 1 significantly prevented the development of morphine antinociceptive tolerance (13.8 ± 0.3 s) compared with the saline-treated group (10 μ L, i.t., n=5: 9.0 ± 0.5 s) (Fig. 1B). Inhibitory effects of 3MA on morphine antinociceptive tolerance exhibited a dose-dependency. Furthermore, wortmannin also inhibited morphine antinociceptive tolerance (Fig. 1C).

Immunoblot analyses revealed that the mean level of Beclin1, a protein that is essential for autophagy (Liang et al., 1999), exhibited a time-dependent increase in the lumbar (L4-5) spinal dorsal horn after morphine administration (Fig. 2A). We further assessed LC3 conversion (soluble type of LC3-I to lipid bound type of LC3-II) after

morphine administration. The mean level of the LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes and required for the formation of autophagosome (Kabeya et al., 2000), significantly increased after morphine administration (Fig. 2B). The morphine-induced increases in the mean levels of both Beclin1 and LC3-II were significantly inhibited by intrathecal injection of 3MA on day 1 (Fig. 2A, B). These results indicate that autophagy is one of the key factors in the development of morphine antinociceptive tolerance.

3.2. Expression patterns of autophagy-related proteins in the superficial spinal cord

Next, we examined the localization of autophagy-related proteins in the spinal cord after chronic morphine administration. Increased expression levels of both Beclin1 and LC3 were observed in the superficial layers of the spinal dorsal horn (lamina I-II) after morphine administration (Fig. 3A-K). Magnified images clearly showed the upregulation of Beclin1 immunofluorescence within the cells. Furthermore, immunofluorescence patterns of LC3 changed from diffuse staining pattern (LC3-I) into dots of punctate pattern (LC3-II) after morphine administration (Fig. 3K, L), indicating a conversion of LC3-I to -II. This was clearly consistent with our immunoblot data (Fig. 2A, B). Morphine-induced increases in the mean IF intensities of Beclin1 and LC3 were significantly inhibited by intrathecal injection of 3MA (Fig. 3C, F, G, J, M, N).

Inhibitory interneurons account for 30-40% of the total neurons distributed in the spinal superficial layer (Todd and Sullivan, 1990). Furthermore, GABAergic neurons account for 30% of the total neurons distributed in the lamina II (Sardella et al., 2011). Thus, the superficial layer neurons play important roles in the gating of pain transmission (Melzack and Wall, 1965). We, therefore, examined a possible induction of

autophagy in the inhibitory GABAergic interneurons using GAD67-GFP knock-in mice. It is notable that GAD67-GFP knock-in mice has been used for the identification of GABAergic neurons in the CNS including spinal dorsal horn (Fukushima et al., 2009, Nowak et al., 2011). Superficial layer GABAergic interneurons were identified by vGluT3 immunoreactivity (Fig. 3O). Immunofluorescences of both Beclin1 and dots signal of LC3, presumably LC3-II, were observed in the GAD67-GFP-positive GABAergic interneurons (Fig. 3P, Q).

3.3. Autophagy increased lysosomal activity after morphine administration

Accumulating evidence has suggested a crosstalk between autophagy and cathepsin B (CatB: EC 3.4.22.1), a lysosomal cysteine protease (Bhoopathi et al., 2010, Ha et al., 2010, Sun et al., 2010, Tatti et al., 2012). Therefore, it is reasonable to consider that CatB is an autophagy-related factor in the development of morphine antinociceptive tolerance. To clarify the autophagy-CatB axis in morphine antinociceptive tolerance, the expression levels of CatB in the spinal cord were examined after morphine administration. Morphine-tolerant mice showed an almost 4-fold increase in the mean IF intensity for CatB in the spinal dorsal horn (Fig. 4A, B). These signals were never detected in CatB-deficient mice 5 days after morphine administration (Fig. 4A, B). Morphine-induced increased IF for CatB was colocalized with those for both Beclin1 and LC3 (Fig. 4C, D) in GAD67-GFP-positive interneurons (Fig. 4E-G), suggesting that CatB is a causative factor for morphine antinociceptive tolerance. Interestingly, the intrathecal administration of 3MA significantly suppressed the morphine-induced increase in the mature CatB in the spinal cord (Fig. 4F). It was also noted that morphine-induced increased IF for CatB was also observed in microglia, but not in

astrocytes, in the dorsal spinal cord (Fig. 5), suggesting a possible involvement of microglial CatB in the morphine antinociceptive tolerance.

3.4. A possible involvement of CatB in the development of morphine tolerance

These observations prompted to further examine a possible involvement of CatB in the development of morphine antinociceptive tolerance. Therefore, effects of gene deletion or pharmacological blockade of CatB on the morphine antinociceptive tolerance were examined. The mean latencies to thermal stimulation in CatB^{-/-} mice were significantly longer than in wild-type mice after morphine administration (Fig. 6A). Furthermore, the intrathecal injection of CA074Me, a membrane-permeable specific CatB inhibitor, dose-dependently suppressed the development of morphine antinociceptive tolerance (Fig. 6B). To further analyze the direct relationship between CatB and autophagy, we analyzed the expression level of Beclin1 in superficial layers of the spinal dorsal horn (lamina I-II) following morphine administration. Beclin1 immunofluorescence was significantly decreased by the presence of intrathecal administration of CA074Me or gene deletion of CatB (Fig. 7 A-E). In addition, Beclin1 immunofluorescence in GAD67-positive neurons was also decreased by CatB inhibitor or CatB-deficient mice (Fig. 7 F-M). These results suggest that CatB is the key molecule of chronic morphine-induced excessive autophagy.

3.5. A possible involvement of CatB in the dysfunction of spinal GABAergic system

Finally, possible changes of spinal GABAergic system associated with morphine antinociceptive tolerance were examined. There was no significant reduction of NeuN- and GAD67-GFP-positive cells in the superficial layer of spinal dorsal horn 5 days after

morphine administration (NeuN: 0.334 ± 0.008 cells/100 μm^2 , GAD67-GFP: 0.080 ± 0.004 cells/100 μm^2) in comparison with those in saline-treated animals (NeuN: 0.333 ± 0.012 cells/100 μm^2 , GAD67-GFP: 0.085 ± 0.005 cells/100 μm^2). On the other hand, chronic morphine administration resulted in a significant increase in the mean frequency of miniature EPSCs (mEPSCs) recorded from lamina I neurons of wild-type mice without affecting the mean amplitude (Fig. 8A, B, E, F). In contrast, neither the mean frequency nor amplitude of mEPSCs recorded from lamina I neurons of CatB^{-/-} mice was significantly changed following chronic morphine administration (Fig. 8C-F). These observations indicate that glutamate release from the presynaptic axon terminals is significantly increased in lamina I neurons of wild-type mice, but not of CatB^{-/-} mice, following chronic morphine administration.

4. Discussion

We investigated a possible role of autophagy in the development of morphine antinociceptive tolerance, because a possible activation of autophagy by morphine has been recently demonstrated (Zhao et al., 2010). We herein showed for the first time the levels of autophagy-related proteins, including Beclin1, LC3 and CatB, were significantly increased in GABAergic interneurons of the superficial layer of the spinal cord following chronic morphine treatment. Moreover, inhibition of autophagy or CatB activity significantly attenuated the development of morphine antinociceptive tolerance.

Interruptions in the autophagic system might lead to deleterious effects on cellular function, because autophagy is involved in the homeostatic system that maintains the intracellular environment (Mizushima and Levine, 2010). In contrast, inhibition of autophagy has sometimes beneficial consequences, because excessive autophagy

induces cell death and dysfunction (Mizushima, 2007, Banerjee et al., 2010). On the other hand, CatB is known as an essential regulator of autophagy in cell death (Ha et al., 2010, Tatti et al., 2013). How 3MA inhibit the upregulation of CatB following morphine administration? The inhibitory mechanism of 3MA on autophagy is known to mediate class III phosphoinositide 3-kinase (PI3K). Beclin1 is to form the PI3K complex. In the present our study, we found that intrathecal administration of 3MA significantly reduced the expression levels of CatB following morphine administration. However, 3MA has no effects on the CatB enzymatic activity (Wu et al., 2010). The mechanism how 3MA suppress the expression level of CatB is as follows. The expression levels of CatB mRNA are promoted by the activation of nuclear factor κ B (NF- κ B) through the binding of NF- κ B subunits p50 and p65 to the NF- κ B binding site in the CatB gene promoter region (Bien et al., 2004). Reactive oxygen species (ROS) are the essential activator of NF- κ B (Morgan and Liu, 2011). It is noteworthy that 3MA can suppress the ROS generation (Liu et al., 2011) besides inhibitory effects of PI3K, indicating that 3MA might indirectly suppress the CatB expression. These facts suggest that CatB is one of important molecules that need to degrade intracellular proteins during autophagy. Therefore, there are at least two possible roles of CatB-dependent autophagy in the development of morphine antinociceptive tolerance through excessive autophagy of GABAergic interneurons in the superficial layer of spinal dorsal horn: (1) neuronal death of GABAergic interneurons, (2) dysfunction of GABAergic interneurons.

The reduction in the number of GAD67-GFP-positive neurons, a key enzyme for the synthesis of GABA, in the superficial layer of the spinal cord dorsal horn diminishes the inhibitory neural circuit of nociceptive neurotransmission. However, morphine tolerance is a phenomenon that is reversible by several inhibitors (Powell et al., 2000,

Shavit et al., 2005, Wang et al., 2012), suggesting that morphine dose not induce lethal injuries to GABAergic interneurons. Our observations here also showed no significant reduction of the mean cell number of neither NeuN- nor GAD67-GFP-positive neurons in the superficial spinal cord after morphine administration. Disinhibition of neural circuit has well known to enhance nociceptive signaling (Mao et al., 2002, Harvey et al., 2004, Coull et al., 2005). Shehata et al. (2012) have reported that prolonged weak neuronal activity triggers a reduction of AMPA receptors on the membrane surface of hippocampal neuron as the consequence of autophagy that is unaccompanied by neuronal death (Shehata et al., 2012). The accumulation of AMPA receptors in autophagosome has also been observed in Purkinje cells (Matsuda et al., 2008). These phenomena account for the reduction in neural activity by autophagy. Substantial evidence has indicated that the reduction of AMPA receptors in the GABAergic interneurons is one of the characteristic alterations in the disinhibition of nociceptive circuits (Kim et al., 2012). Another noteworthy alteration has observed in the synaptic terminal. Rapamycin, an autophagy inducer, increase the autophagosomes in the synaptic terminal in the striatum where the number of synaptic vesicles and dopamine release have reduced (Hernandez et al., 2012). The impairment of autophagic degradation was due to the reduced enzymatic activity of CatB. Consequently, CatB might degrade synaptic vesicle in GABAergic interneuron through autophagy. Our observations here showed that there was a significant increase in the mean frequency of mEPSCs recorded from lamina I neurons of wild-type mice without affecting the mean amplitude, but not in CatB^{-/-} mice, following chronic treatment of morphine. It is generally accepted that GABAergic interneurons in lamina I-II make synapses with C-fiber terminals (Todd and Lochhead, 1990). GABA inhibits the release of glutamate

and neuropeptide from C-fiber through the presynaptic GABA_B receptors (Malcangio and Bowery, 1996). These observations suggest that CatB increases glutamate release from the primary afferent through dysfunction of GABAergic interneurons following chronic morphine treatment. Therefore, the involvement in the dysfunction of GABAergic interneurons is a more likely role of CatB in the development of morphine antinociceptive tolerance.

The mechanism how CatB was topically upregulated in the superficial layer of spinal cord remains unclear. It is conceivable that upregulated expression of CatB was dependent on the mu opioid receptors because morphine-induced autophagy in the hippocampal neurons is mediated by mu opioid receptor (Zhao et al., 2010). Evidences indicated that mu opioid receptors express GABAergic interneurons in the several regions (Drake and Milner, 2002, Huo et al., 2005, Kudo et al., 2014). In contrast, a few population of GABAergic neurons express mu opioid receptors in the superficial layer of spinal dorsal horn (Kemp et al., 1996). There are controversial actions of mu opioid receptor on GABAergic neurons. Kerchner et al., showed that mu opioid receptor activation reduced evoked IPSC in cultured spinal dorsal horn (Kerchner and Zhuo, 2002). On the other hand, Kohno et al., showed that [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO), a mu opioid receptor agonist, has no effect on inhibitory neurotransmission in acute slice preparations of lumbar spinal cord (Kohno et al., 1999). In the future study, we will analyze the localization of mu opioid receptor on GABAergic neuron with patch clamp analyses using GAD67-GFP knockin mice. Then, we will conclude whether CatB upregulation is mediated by mu opioid receptors.

The increased expression of CatB was not restricted to neurons. We also detected the upregulation of CatB in the spinal microglia 5 days after morphine administration.

Increasing evidence has suggested the significance of microglia in morphine tolerance (Horvath et al., 2010, Zhou et al., 2010). IL-1 β , an inflammatory cytokine, has one of the major roles in morphine tolerance (Shavit et al., 2005). Interestingly, the pharmacological blockade or gene deletion of CatB significantly suppressed the expression levels of the mature type of IL-1 β in spinal microglia in morphine-tolerant animals (data not shown). We have previously reported that the processing of IL-1 β is dependent on the CatB through caspase-1 activation in chromogranin A-stimulated microglia (Terada et al., 2010). This pathway is part of the molecular machinery of inflammatory pain (Sun et al., 2012). In addition, glia-derived proinflammatory cytokines, such as IL-1 β and IL-6, can suppress inhibitory neurotransmission in the spinal dorsal horn (Kawasaki et al., 2008). Microglia-derived IL-1 β affects the synthesis of prostaglandin E₂ through the induction of cyclooxygenase-2 (Samad et al., 2001), which might cause a marked reduction in inhibitory input (Harvey et al., 2004). Ferrini et al. (2013) found that brain-derived neurotrophic factor from microglia is involved in the development in the pain hypersensitivity after chronic morphine administration (Ferrini et al., 2013). Therefore, increased CatB in spinal microglia are also involved in the development of morphine antinociceptive tolerance.

5. Conclusions

In the present study, chronic treatment of morphine induces CatB-dependent excessive autophagy in the GABAergic interneurons in the superficial layer of spinal dorsal horn. It is considered that CatB-dependent excessive autophagy is directly involved in the development of morphine antinociceptive tolerance, because inhibition of autophagy or CatB activity significantly attenuated its development. Our

observations propose that a dysfunction of GABAergic interneurons through CatB-dependent excessive autophagy is an important causative factor for the development of morphine antinociceptive tolerance. Therefore, autophagy or CatB inhibitors can be beneficial in the blockade of opioid antinociceptive tolerance.

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Legends to Figure

Figure 1

Effects of an autophagy inhibitor on morphine antinociceptive tolerance. (A)

Morphine administered intraperitoneally on day 1. Hot-plate latency was examined 1 h after morphine (10 mg/kg) administration. The data are mean $\pm$ s.e.m. (n=5 animals each), *** P < 0.001: two-way repeated measurements analysis of variance (ANOVA).

(B) Inhibitory effects of 3-methyladenine (3MA) on the development of morphine tolerance. Arrow indicates the intrathecal (i.t.) injection of saline (10 μ L) or 3-methyladenine (3MA; 1, 3, 10, or 30 μ g /10 μ L) that was conducted for 1 h prior to morphine administration. The data are mean $\pm$ s.e.m. (n=5-9 animals each), ** P < 0.01, *** P < 0.001; Saline vs 3MA 30 μ g, ††† P < 0.001; Saline vs 3MA 10 μ g, # P < 0.05, ## P < 0.01; Saline vs 3MA 3 μ g: two-way repeated measurements ANOVA. (C) Wortmannin suppressed morphine tolerance. Arrow indicates the i.t. injection wortmannin (0.1, 0.3, or 1 μ g/10 μ L). The data are mean $\pm$ s.e.m. (n=9 animals each), *** P < 0.001; Saline vs wortmannin 1 μ g, ††† P < 0.001; Saline vs 0.3 μ g, # P < 0.05; Saline vs wortmannin 0.1 μ g: two-way repeated measurements ANOVA.

Figure 2

Chronic morphine administration initiates autophagy in the spinal dorsal horn.

Time-dependent induction of Beclin1 (A) and the conversion of LC3 (soluble LC3-I to lipid bound LC3-II) (B) after morphine administration. The data are mean $\pm$ s.e.m. (n=3 animals each). ** P < 0.01, *** P < 0.001 (vs. saline), ††† P < 0.001 (vs. 5d); one-way ANOVA.

Figure 3

Localization of autophagy-related protein after morphine administration. (A-N) The increased levels of Beclin1 or LC3 immunofluorescence 5 days after morphine administration were elevated in the superficial layer of spinal cord. The intrathecal injection of 3MA significantly suppressed upregulation of Beclin1 and conversion of LC3 after morphine administration. A solid line indicated the surface of the gray matter. A broken line indicated the border of lamina II and III which was identified by vGluT3 immunofluorescence. The data are mean $\pm$ s.e.m. (n=6 slices from 3 animals each). * $P < 0.05$, ** $P < 0.01$; one-way ANOVA. (O) Immunofluorescence of GFP (green) and vGluT3 (red) in the spinal cord of GAD67-GFP knock-in mice. Inset indicated the single image of vGluT3. (P, Q) Immunofluorescence of Beclin1 (P) or LC3 (Q) (red) in superficial layer of GABAergic interneuron of the morphine-tolerant GAD67-GFP knock-in mice. Scale bar = 50 μ m (A-C, H-J, O), 10 μ m (D-F, K-M, P, Q). Magnification: $\times 20$ (A-C, H-J, O), $\times 100$ (D-F, K-M, P, Q).

Figure 4

Involvement of CatB in the development of morphine antinociceptive tolerance. (A) CatB immunofluorescence was upregulated 5 days after morphine administration in the wild-type but not in the cathepsin B-deficient (CatB^{-/-}) mice. A solid line indicated the surface of the gray matter. A broken line indicated the border of lamina II and III. Scale bar = 50 μ m. (B) The data are mean $\pm$ s.e.m. (n = 4-5 slices from 3 animals each). *** $P < 0.001$; one-way ANOVA. (C, D) Colocalized signal (arrowhead) of Beclin1 (C) and LC3 (D) with CatB (green) in morphine-tolerant animals. (E) CatB

immunofluorescence (red) was detected in the GABAergic interneuron of spinal dorsal horn of morphine tolerant GAD67-GFP knock-in mice. Scale bar= 50 μ m (A), 10 μ m (C-E). (F) Immunoblot for the mature type of CatB (mCatB) after morphine administration. The data are mean $\pm$ s.e.m. (n=3 animals each). * P < 0.05, ** P < 0.01 (vs. saline), † P < 0.05 (vs. 5d); one-way ANOVA. Magnification: $\times$ 20 (A), $\times$ 100 (C-D).

Figure 5

Distribution of CatB in the spinal dorsal horn. (A-C) Double immunofluorescence of CatB (green) and Iba1 (microglia), NeuN (neuron), or GFAP (astrocyte) in the morphine-tolerant mice. CatB predominantly express in the microglia and neuron. In contrast, astrocyte have little CatB immunoreactivity. Magnification: $\times$ 20.

Figure 6

Inhibition of CatB activity prevented the development of morphine antinociceptive tolerance. (A) CatB-deficiency was resistant to the development of morphine antinociceptive tolerance. The data are mean $\pm$ s.e.m. (n = 5 animals each). * P < 0.05, ** P < 0.01, (closed circle vs. open square); two-way ANOVA. (B) Intrathecal administration of CA074Me, a membrane-permeable CatB inhibitor, dose-dependently suppressed morphine antinociceptive tolerance. The data are mean $\pm$ SEM. (n = 5 animals each). * P < 0.05, ** P < 0.01, *** P < 0.001, (closed squares vs. closed circles). †† P < 0.01, ††† P < 0.001, (open diamonds vs. closed circles); two-way ANOVA.

Figure 7

Inhibition of CatB activity prevented the induction of Beclin1 following morphine administration. (A-E) Increased Beclin1 immunofluorescence 5 days after morphine administration was significantly suppressed by CatB inhibitor or CatB-deficient (CatB^{-/-}) mice. A solid line indicated the surface of the gray matter. A broken line indicated the border of lamina II and III. Scale bar = 50 μ m. Insets indicate vGluT3 immunoreactivity. The data are mean $\pm$ s.e.m. (n = 4-5 slices from 3 animals each). *** $P < 0.001$; one-way ANOVA. (F-M) CatB-inhibitor or gene deletion of CatB suppressed the Beclin1 immunofluorescence (J-M) within GAD67-positive neuron (F-I). Beclin1 immunofluorescence (J-M) within GAD67-positive neuron (F-I) increased following morphine administration. Scale bar= 50 μ m (A-D), 10 μ m (F-M). Magnification: $\times 20$ (A-D), $\times 100$ (F-M).

Figure 8

Suppressive effects of CatB-deletion on morphine-induced enhanced neurotransmission. Voltage clamp recordings from lamina I neurons of wild-type (A, B) or CatB^{-/-} (C, D) mice 5 days after morphine administration. The mean frequency (E) and amplitude (F) of miniature excitatory postsynaptic currents (mEPSCs) that were obtained from lamina I neurons. The data are mean $\pm$ s.e.m. (N = 4 animals, n = 5-8 neurons each from 3 animals), * $P < 0.05$; ** $P < 0.01$; two-way ANOVA.

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 8