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Morphine induces Beclin 1- and *ATG5*-dependent autophagy in human neuroblastoma SH-SY5Y cells and in the rat hippocampus

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Key words: morphine, autophagy, cell death, Beclin 1, *ATG5*

Abbreviations: EGFP, enhanced green fluorescent protein; LC3, microtubule-associated protein light chain 3; BAF A1, bafilomycin A1; PTX, pertussis toxin; *ATG5*, autophagy-related gene 5; *ATG7*, autophagy-related gene 7; Bcl-2, B-cell lymphoma/leukemia-2; Bcl-x_L, B-cell lymphoma extra long; Co-IP, co-immunoprecipitation; EBSS, earle's balanced salt solutions

Chronic exposure to morphine can induce drug addiction and neural injury, but the exact mechanism is not fully understood. Here we show that morphine induces autophagy in neuroblastoma SH-SY5Y cells and in the rat hippocampus. Pharmacological approach shows that this effect appears to be mediated by PTX-sensitive G protein-coupled receptors signaling cascade. Morphine increases Beclin 1 expression and reduces the interaction between Beclin 1 and Bcl-2, thus releasing *Beclin 1* for its pro-autophagic activity. Bcl-2 overexpression inhibits morphine-induced autophagy, whereas knockdown of Beclin 1 or knockout of *ATG5* prevents morphine-induced autophagy. In addition, chronic treatment with morphine induces cell death, which is increased by autophagy inhibition through *Beclin 1* RNAi. Our data are the first to reveal that Beclin 1 and ATG5 play key roles in morphine-induced autophagy, which may contribute to morphine-induced neuronal injury.

Introduction

Morphine is clinically used for pain relief in cancer patients. However, chronic exposure of morphine can induce drug addiction, gross impairment of dopaminergic neurons and neural injury.[1] Indeed, numerous reports show that morphine induces brain damage and neuronal toxicity by inhibiting cell growth and inducing apoptosis both in vitro[2-4] and in vivo.[5-7] These inhibitory effects may be G protein-dependent following the engagement of morphine with the opioid receptors in SK-N-SH cells.[8] Morphine promotes apoptosis in macrophages and in Jurkat T cells through oxidative stress, which subsequently activates cell death pathways,[4] or through production of TGF-β,[9] which may contribute its effect on immune suppression. In contrast to these reports, there are also studies showing that morphine can have protective effects against cell death.[10,11] Morphine prevents peroxynitrite-induced death of SH-SY5Y cells through a direct scavenging action,[12] and it even stimulates cell growth in mouse retinal endothelial cells.[13] It appears that the effects of morphine on cell death are cell type-dependent, and the exact mechanism of morphine-induced neurotoxicity remains a subject of debate.

Autophagy is a regulated cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosomes. In neurons, a constitutive, basal level of autophagy helps to control the cellular quality of proteins[14] and protects cells from protein aggregation[15] or damaged organelles.[16] Autophagy can be activated in response to environmental cues such as nutrient depletion and temperature and oxidative stresses.[17] Autophagy is highly regulated by the *ATG* genes such as *Beclin 1*,[18] *ATG5*,[19] and *ATG7*.[20] Beclin 1 is a phylogenetically conserved protein that is essential for the initiation of autophagy.[21] Beclin 1 interacts with numerous partners such as UVRAG, Ambra1 and Bif-1 in the initiation of autophagosome formation.[22] This process is strongly inhibited by Bcl-2 and Bcl-x_L, the key anti-apoptotic Bcl-2 family proteins.[23] The interaction of Beclin 1 with Bcl-2 plays a key role for the regulation of autophagy in addition to Bcl-2's established role in apoptosis.[24] In contrast to its protective effect, autophagy can also be a cell death mechanism.[25] Ischemia/hypoxia,[26] oxidative stress[27] and some chemical reagents, such as methamphetamine,[28] tryptamine[29] and dopamine,[30] induce autophagic cell death in neuronal cell lines or normal neurons. We herein address the possibility that morphine may activate autophagy which may

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result in autophagy-associated cell death of neuronal cells. Our data are the first to reveal that Beclin 1 and ATG5 play key roles in morphine-induced autophagy. Better understanding of the mechanisms of action of morphine holds promise for better management of cancer patients and morphine use.

Results

During autophagy, the cytoplasmic form of LC3 (LC3-I, 18 kDa) is converted to the preautophagosomal and autophagosomal membrane-bound form (LC3-II, 16 kDa).[32] LC3 is thus used as a specific marker for autophagosome formation, although there are some limitations as it is tissue- and cell-dependent. Transient overexpression of GFP-LC3 is not used as LC3 aggregates are often formed within cells.[33-35] To determine whether morphine could induce autophagy in neuronal cells, we treated the pEGFP-LC3 stably transfected SH-SY5Y cells with morphine hydrochloride and found that there were increased numbers of punctate GFP-LC3 dots in the treated cells in a time- and dose-dependent manner (**Fig. 1A and B, and Fig. S1**). Western blot analysis revealed a steadily increasing quantity of the LC3-II form in morphine-treated SH-SY5Y cells (**Fig. 1C and D**). As increased LC3-II levels can occur when autophagy is either induced or inhibited,[33] lysosomal inhibitor, bafilomycin A1 (BAF A1), which prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes, was used to determine that morphine-induced LC3-II levels increased as a result of increased autophagosome formation rather than a defect in the fusion process. Results showed that BAF A1, significantly increased LC3-II levels (**Fig. 1C and D**) and autophagosome formation (**Fig. 1B**). Autophagy is characterized by the formation of double-membraned autophagosomes that fuse with lysosomes to form autolysosomes and undergo degradation.[36] To confirm that morphine induces autophagy, we examined the ultrastructure of the cells by electron microscopy and found abundant vacuolar elements which are most likely to be of autophagic origin in SH-SY5Y cells after treatment with 200 μM morphine for 24 h (**Fig. 1E**). Collectively, our results clearly demonstrated that morphine induces an autophagic response in SH-SY5Y cells (**Fig. 1**).

Morphine activates a receptor-mediated G protein-coupled signaling pathway upon engagement with its receptors. We therefore asked whether morphine-induced autophagy is mediated by opioid receptors. We first used a pharmacological approach to address this question. Naloxone is a general antagonist of opioid receptors, and previous reports showed that it blocks the effects of morphine.[37] Our results show that pretreatment of SH-SY5Y cells with naloxone (100 μM) reduced the morphine-induced increase in LC3-II levels (**Fig. 2A**). We next used PTX and suramin, two antagonists of G protein signaling pathways, and found that both PTX (100 ng/ml) and suramin (100 μM) completely blocked the increase in LC3-II levels (**Fig. 2B**). These results provide strong evidence to suggest that morphine induces autophagy through an opioid receptor-mediated and PTX-sensitive G protein pathway.

Beclin 1 is one of the key mediators in the formation of the autophagosome,[18] as it is involved in the initial step of autophagosome formation.[38] We hypothesized that Beclin 1 is also important for morphine-induced autophagy. Consistent with this hypothesis, we found that Beclin 1 is upregulated in morphine-treated SH-SY5Y cells (**Fig. 3A**). In contrast, the expression of Bcl-2, Bcl-x_L, two Beclin 1-interacting proteins in the Bcl-2 family,[39] was not affected. The pro-apoptotic Bcl-2 protein Bax, also remained unchanged. It has been reported that Beclin 1 is released from Bcl-2 during the initiation of autophagy.[24,40] We therefore determined whether morphine disrupts the interaction between Beclin 1 and Bcl-2, thereby initiating autophagy. Indeed, the results of co-immunoprecipitation (Co-IP) with anti-Bcl-2 antibody and blotting with anti-Bcl-2 and anti-Beclin 1 antibodies showed that the interaction of Beclin 1 with Bcl-2 progressively decreased (**Fig. 3B and C**). To further substantiate the role of Bcl-2 in the regulation of autophagy, we found that Bcl-2 overexpression significantly reduced the morphine-induced increase in LC3-II levels (**Fig. 3D**) and increased the binding with Beclin 1 (**Fig. 3E**). To confirm the function of Beclin 1 in morphine-induced autophagy, we used a shRNA expressed in the p*Silencer* 2.1-U6 Hygro vector, which specifically knocked down Beclin 1 expression in SH-SY5Y cells. As expected, the level of autophagy induced by morphine, as shown by LC3-II levels, was decreased by *Beclin 1* suppression (**Fig. 3F**). Collectively, these data demonstrate that morphine induces Beclin 1 release from Bcl-2 and that the released Beclin 1 plays a key role in morphine-induced autophagy.

In addition to Beclin 1, ATG5 has been reported to regulate autophagy;[19] therefore, we next examined ATG5's role in morphine-induced autophagy. We found that morphine completely failed to induce autophagy in *ATG5*-knockout MEFs, whereas wild-type MEFs showed pronounced autophagy upon morphine treatment, although less than that in SH-SY5Y cells (**Fig. 4A**). Unlike Beclin 1, however, the level of ATG5 protein remained unchanged with morphine treatment (**Fig. 4B**). These results showed that ATG5 is also important in morphine-induced autophagy.

All the data clearly show that morphine induces autophagy in SH-SY5Y cells. We next asked whether morphine might induce autophagy in the rat brain. Our results showed that either chronic or acute morphine treatment increased LC3-II levels in the hippocampus; however, we found no detectable effect of morphine on LC3-II levels in the striatum (**Fig. 5A**). This result indicates that morphine-induced autophagy may be cell-type specific in the brain. Naloxone also inhibited the morphine-induced increase in LC3-II levels in the rat hippocampus (**Fig. 5B**), confirming that morphine-induced autophagy is mediated through the opioid receptors.

Accumulating evidence has shown that autophagy has dual roles in cell death.[16,36,41,42] Some results suggest that autophagy in neurons provides a neuroprotective mechanism,[43,44] however, some reports show that autophagy is harmful.[26,27] Increasing evidence suggests that the effects of autophagy are highly contextual.[45,46] We therefore asked whether morphine-induced autophagy has a protective or a harmful role in the neural system. Cell death detection showed that higher dose (500, 1,000 μM at 48 h) or longer time (200 μM at 72 h) of morphine treatment could induce cell death (**Fig. 6A**), which was increased when

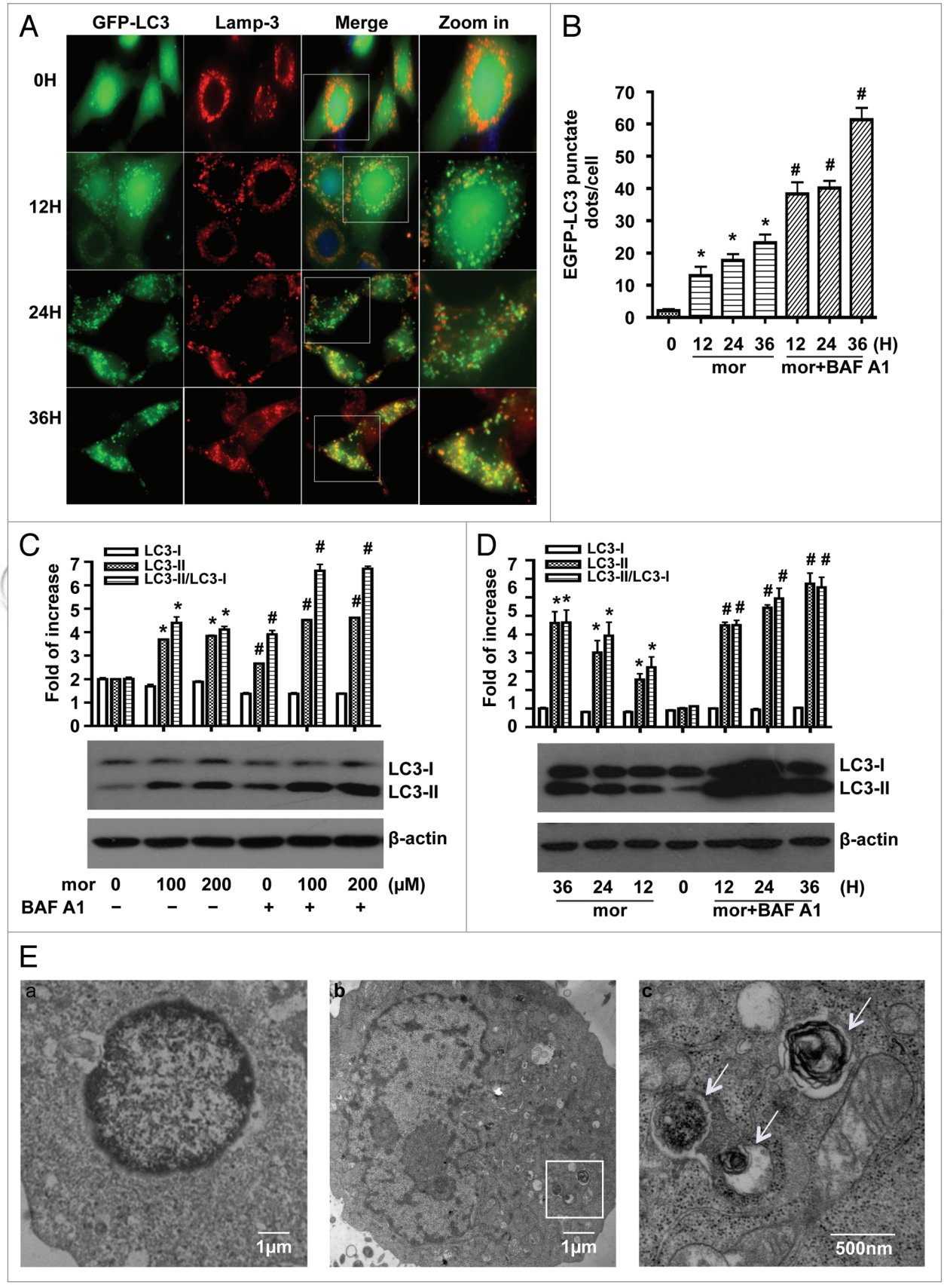

Figure 1. For figure legend, see page 389.

Figure 1 (See opposite page). Morphine induces autophagy in SH-SY5Y cells, in a time- and dose-dependent manner, and is increased by bafilomycin A1. (A) Punctate GFP-LC3 dots in morphine-treated SH-SY5Y cells. pEGFP-LC3 stably transfected SH-SY5Y cells were treated with 200 μM morphine for the indicated times. Cells were fixed with formaldehyde (3.7% w/v) and immunostained with anti-Lamp-3 antibody for detecting lysosomes. Cells were examined by fluorescence confocal microscopy (X63/oil). (B) pEGFP-LC3 stably transfected SH-SY5Y cells were treated with 200 μM morphine for the indicated times, with or without 20 nM BAF A1. Punctate GFP-LC3 dots in cells were counted. Data were the mean value of three independent experiments with each count of no less than 200 cells. *$p < 0.01$ as compared with control. #$p < 0.01$ as compared with morphine alone. (C and D) LC3-II was significantly increased in morphine-treated SH-SY5Y cells, and was increased by BAF A1. (C) SH-SY5Y cells were exposed to 100 or 200 μM morphine for 12 h, with or without 20 nM BAF A1 and then subjected to western blot analysis with anti-LC3 antibody. Positions of LC3-I and LC3-II are indicated. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *$p < 0.001$ as compared with control. #$p < 0.001$ as compared with morphine alone. (D) SH-SY5Y cells treated with 200 μM morphine for the indicated times, with or without 20 nM BAF A1 were also subjected to western blot analysis. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *$p < 0.01$ as compared with control, #$p < 0.01$ as compared with morphine alone. (E) Electron micrographs showing the ultrastructure of morphine-treated SH-SY5Y cells. (a) Control (untreated SH-SY5Y cells), (b and c) SH-SY5Y cells treated with 200 μM morphine for 24 h. Arrows in the electron micrograph denote representative presumed autophagic bodies.

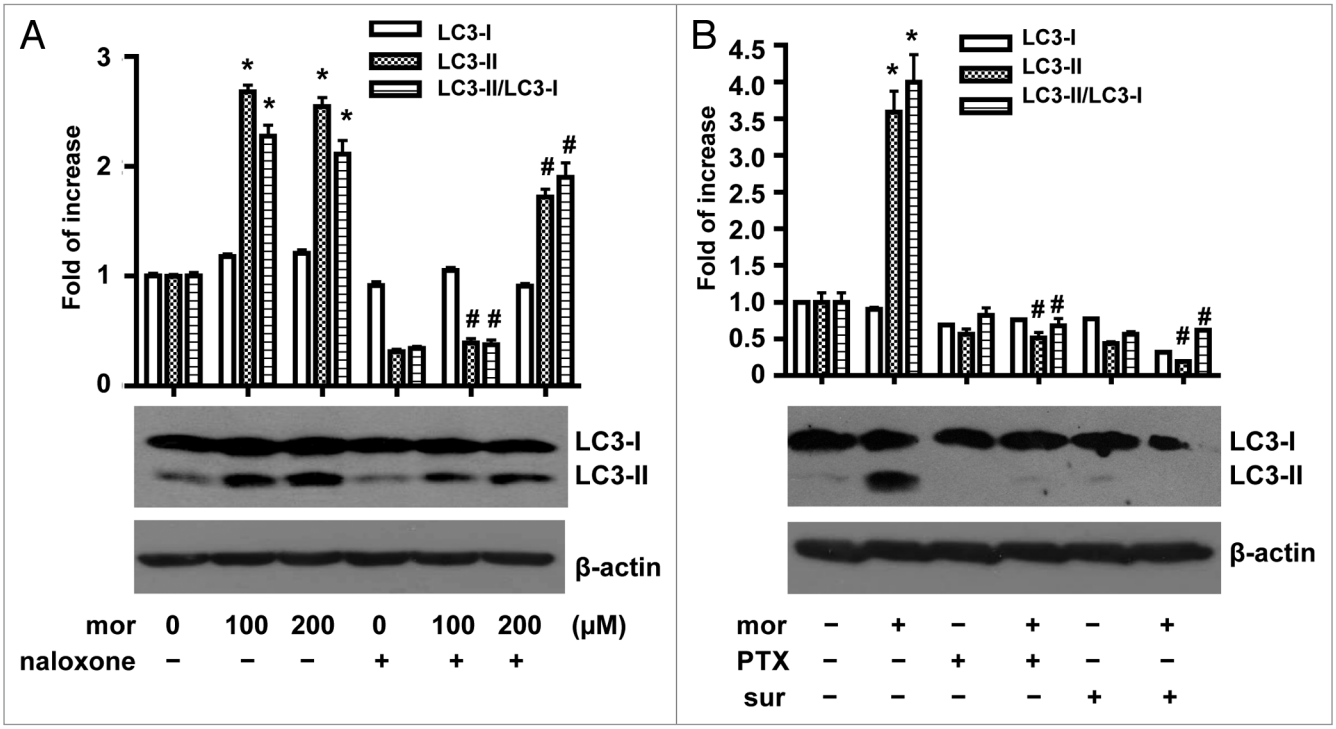

Figure 2. Morphine induces autophagy through an opioid receptor-mediated PTX-sensitive G protein pathway. (A) SH-SY5Y cells were treated with or without 100 μM naloxone for 30 minutes and then treated with different concentrations of morphine for 12 h. LC3-I and LC3-II were detected by western blot analysis. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *$p < 0.001$ as compared with control. #$p < 0.001$ as compared with morphine alone. (B) SH-SY5Y cells were treated with or without 100 ng/ml PTX or 100 μM suramin for 30 minutes, after which 200 μM morphine was added for 12 h. LC3-I and LC3-II were detected by western blot analysis. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *$p < 0.001$ as compared with control. #$p < 0.001$ as compared with morphine alone.

autophagy was inhibited by *Beclin 1* knockdown (**Fig. 6B and C**). The results that cell death was increased by autophagy inhibition indicated that autophagy is an early response to the morphine induced-stress and may have a protective role in cell death; however, chronic exposure leads to extensive autophagy which may damage the cellular components leading towards cell death.

Discussion

Morphine is widely used clinically for pain management in cancer patients. Chronic exposure of morphine could induce drug addiction and neural injury. The exact mechanism is not fully understood. The present study revealed that the exposure to morphine induces autophagy in SH-SY5Y cells and in the rat hippocampus. Autophagy is an early event following the treatment of morphine; it is found that autophagy could be detected as early as 0.5 h with morphine treatment (**Fig. S1B**). Accumulating evidence suggests that autophagy-associated cell death or type II programmed cell death,[41] can occur in some cell types, but the role of autophagy in morphine-induced cell death was not previously explored. We detected cell death (**Fig. 6**) in SH-SY5Y cells at later time points and cell death was increased by autophagy

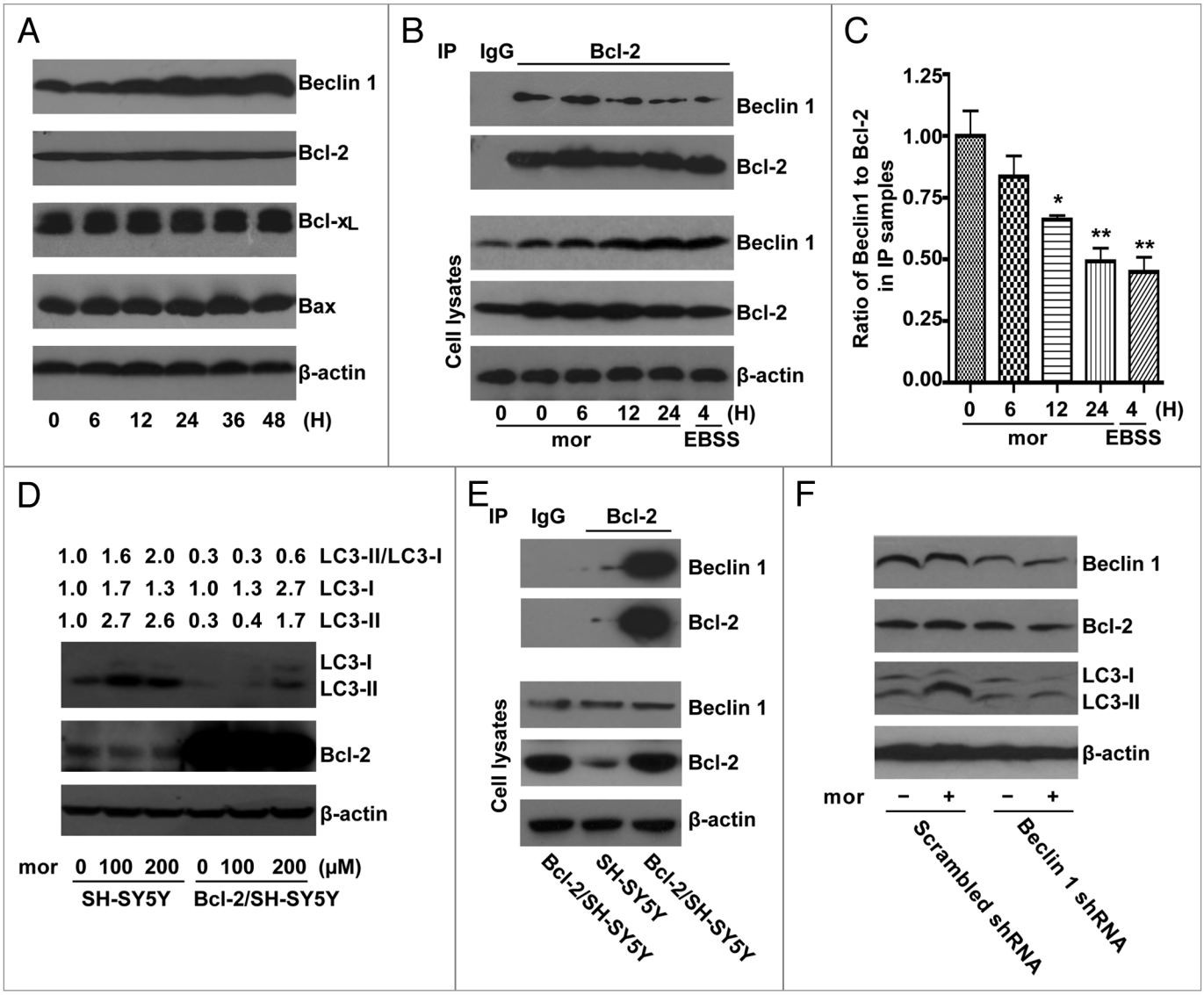

Figure 3. Beclin 1 is important for morphine-induced autophagy. (A) Expression of Beclin 1 and other proteins in morphine-treated SH-SY5Y cells. SH-SY5Y cells were exposed to 200 μM morphine for the indicated times, then subjected to western blot analysis using anti-Beclin 1, anti-Bcl-2, anti-Bcl-x_L and anti-Bax antibodies. (B) Morphine attenuates the interaction between Beclin 1 and Bcl-2, and releases Beclin 1. SH-SY5Y cells were treated with 200 μM morphine for the indicated times and then subjected to co-immunoprecipitation analysis. Immunoprecipitation of Beclin 1 and Bcl-2 with Bcl-2 antibody and the level of Beclin 1 and Bcl-2 were detected. Control SH-SY5Y cells immunoprecipitated with IgG as a negative control. SH-SY5Y cells cultured in EBSS (Earle's Balanced Salt Solutions) for 4 h were used as a positive control. (C) The data in panel B were quantified by Image J software. Results were shown as average ± SD for three separate experiments. *$p < 0.05$, **$p < 0.01$ as compared with control. (D) SH-SY5Y or Bcl-2/SH-SY5Y cells were treated with 100 or 200 μM morphine for 12 h and then LC3-I and LC3-II were detected by western blot analysis. The data were quantified by Image J software. The ratio of LC3-I, LC3-II and LC3-II/LC3-I versus control is shown. (E) Bcl-2 overexpression increases the interaction of Bcl-2 with Beclin 1. Immunoprecipitation of Beclin 1 and Bcl-2 with Bcl-2 antibody and the level of Beclin 1 and Bcl-2 were detected in SH-SY5Y and Bcl-2/SH-SY5Y cells. (F) Beclin 1 RNAi inhibits morphine-induced autophagy. Hygromycin-selected SH-SY5Y cells transfected with a *Beclin 1* shRNA plasmid or a scrambled shRNA plasmid were treated with 200 μM morphine for 24 h, and then LC3-I and LC3-II were detected by western blot analysis.

inhibition by knockdown of *Beclin 1*. These data suggest that autophagy is an early response to the morphine induced-stress and may have a protective role in cell death; however, chronic exposure leads to extensive autophagy which may damage the cellular components leading towards cell death. All of these results may help to explain how chronic exposure of morphine may contribute to neural injury.

It is well recognized that morphine could bind to opioid receptors leading to the activation of the G protein-coupled receptor mediated pathway. Our results showed that autophagy induced by morphine is mediated by opioid receptors in a G protein-dependent manner. We showed that naloxone, a general antagonist of opioid receptors, and PTX and suramin, two G protein-coupled receptor antagonists, strongly inhibited

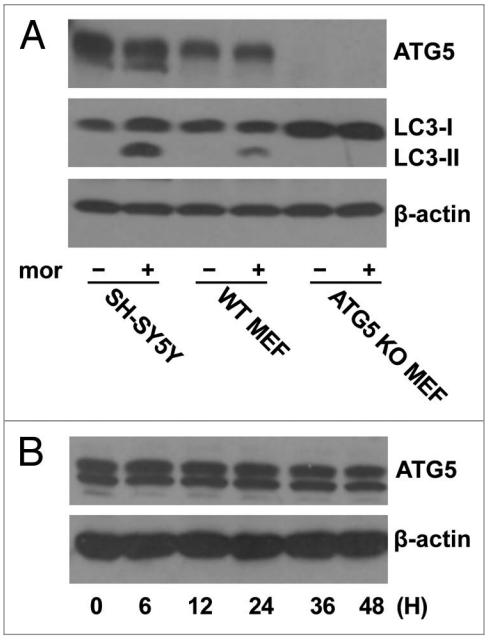

Figure 4. *ATG5*-knockout inhibits morphine induced-autophagy. (A) SH-SY5Y and wild-type and *ATG5*-knockout MEF cells were treated with morphine for 24 h and then LC3-I and LC3-II were detected by western blot analysis. (B) ATG5 protein expression in morphine-treated SH-SY5Y cells. SH-SY5Y cells were exposed to 200 μM morphine for the indicated times and then subjected to western blot analysis with anti-ATG5 antibody.

morphine-induced autophagy. The exact mechanism through which the G protein-coupled signaling pathway is linked with autophagic machinery needs to be further elucidated. Our data revealed that the interaction between Beclin 1 and Bcl-2 may regulate autophagy induced by morphine. Supporting this, we found that the interaction between Beclin 1 and Bcl-2 is reduced following treatment with morphine, and Bcl-2 overexpression blocks morphine-induced autophagy. In addition, knockdown of *Beclin 1* by shRNA reduces autophagy in SH-SY5Y cells. It would be interesting to examine if the activation of G protein pathway is directly related to the increase of Beclin 1. Also, we considered the possibility that JNK, which can be activated by a G protein signaling pathway,[48,49] can phosphorylate Bcl-2 and thereby attenuate the interaction of Beclin 1 and Bcl-2, enhancing autophagy. However, the JNK inhibitor SP600125 appears not to prevent morphine-induced autophagy in SH-SY5Y cells. We found that ROS production is increased in morphine-treated SH-SY5Y cells and that the ROS scavenger, NAC, blocks ROS production and morphine-induced autophagy (**Fig. S3**). Thus, the increase of ROS induced by morphine may promote autophagy.

It is interesting to note that LC3-II increase is detected in the hippocampus but not in other regions of the brain. Our study provides evidence that morphine exerts its toxic effects through the induction of autophagy in the rat brain. The results are consistent with a previous study which shows that chronic exposure to morphine dramatically alters neuronal phenotypes in the dentate gyrus-CA3 region of the adult rat hippocampus,[50] although autophagy was not documented in this study. Why the hippocampus region is the most affected region in the brain remains to be determined. It has been reported that a high dose of dopamine induces autophagic cell death in SH-SY5Y cells.[30] There may be interplay between the morphine-activated signaling pathway and dopamine-related signaling pathways for autophagic cell death. Hypoxic-ischemic injury induces a dramatic increase in autophagosome formation and extensive hippocampus neuron death, but mice deficient in Atg7 are defective for autophagy and neuron death, suggesting that autophagy is causally linked with cell death.[51] Further work will address how morphine leads to neuronal cell death in the absence of *ATG7* or other *ATG* genes.

Materials and Methods

Materials. Morphine hydrochloride was purchased from Qinghai Company, China. pEGFP-C1-LC3 plasmid was kindly provided by Dr. Noboru Mizushima (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The rabbit anti-LC3 polyclonal antibody was kindly provided by Dr. Tamotsu Yoshimori (National Institute of Genetics, Shizuoka-ken, JAPAN) and Dr. Yingyu Chen (Peking University Health Science Center, Beijing, China) and was purchased from Sigma (L7543). Anti-β-actin (monoclonal, A5316) and anti-ATG5 (polyclonal, A0856) antibodies were purchased from Sigma. Anti-Beclin 1 (monoclonal, 612113) and anti-Bcl-2 (monoclonal, 610539) antibodies were purchased from BD Transduction Labs. Anti-Bcl-x_L (polyclonal, 56361) antibody was purchased from BD PharMingen. Anti-Bax (polyclonal, sc-493) antibody was purchased from Santa Cruz Biotechnology. Secondary antibodies (HRP-labeled Goat Anti-Mouse IgG, 074-1806; HRP-labeled Goat Anti-Rabbit IgG, 074-1506) were purchased from KPL, Kirkegaard & Perry Laboratories. Enhanced chemiluminescence (ECL) reagents (WBKLS0500) were purchased from Millipore. All other chemicals were purchased from Sigma unless otherwise specified.

Cell culture, transfection and beclin 1 RNAi. SH-SY5Y, wild-type and *ATG5*-knockout mouse embryonic fibroblast (MEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM, 12100-046; Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS, SH30088.03; Thermo Scientific HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were maintained in a humidified 10% CO_2 atmosphere at 37°C. SH-SY5Y cells at subconfluency were transfected with pEGFP-C1-LC3 plasmid DNA using Lipofectamine™ 2000 Reagent (11668-019, Invitrogen Corporation), following the procedure recommended by the manufacturer and then selected with 600 μg/ml geneticin. A human *Beclin 1* shRNA hairpin (target sequence: CTC AGG AGA GGA GCC ATT T) was cloned into the HindIII and BamH1 sites of p*Silencer* 2.1-U6 Hygro. Beclin 1 shRNA and control scrambled shRNA plasmids were transfected into SH-SY5Y cells and selected with 50 μg/ml Hygromycin.

Fluorescence confocal microscopy. pEGFP-LC3 stably transfected SH-SY5Y cells were cultured on coverslips and then treated with or without morphine for the indicated times. The coverslips were mounted in Permeafluor Aqueous mounting medium

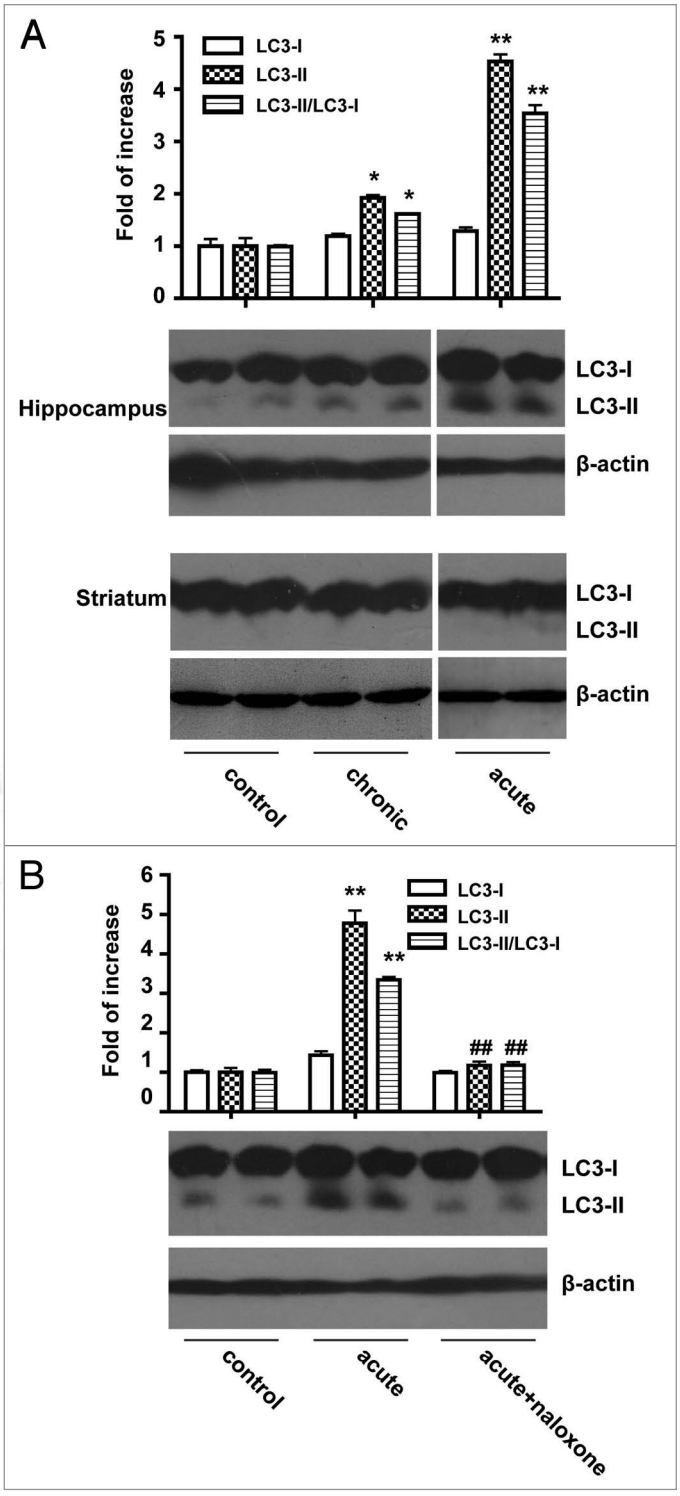

Figure 5. Morphine induces autophagy in the rat hippocampus. (A) The hippocampuses and striatums from control, "chronic" and "acute" groups of rats were lysed with NP-40 buffer, and then LC3-I and LC3-II were detected by western blot analysis. The data were quantified by Image J software. Results were shown as average ± SD for three separate experiments. *p < 0.05, **p < 0.01 as compared with control. (B) The hippocampuses of the three groups of rats were lysed in NP-40 buffer, and then LC3-I and LC3-II were detected by western blot analysis. The data were quantified by Image J software. Results were shown as average ± SD for three separate experiments. **p < 0.01 as compared with control. ##p < 0.01 as compared with "acute" morphine treatment.

(Immunon) and examined by confocal microscopy using the Zeiss LSM 510 META.

Electron microscopy. SH-SY5Y cells were treated as indicated and fixed with 2.5% glutaraldehyde (diluted in DMEM) for 15 min. The samples were then washed thoroughly with PBS and fixed in 1% Os_2O_4 for 2 h at 4°C. After dehydration, samples were embedded in Spur™ for 24 h at 65°C. After being stained with uranyl acetate and lead citrate, the sections were observed under a transmission electron microscope (JEOL-1010). Images were collected by Optronics MicroFire CCD Camera.

Protein expression analysis. Western blotting was performed as described previously.[31] Briefly, cells were washed in PBS and lysed with buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM DTT, and Protease Inhibitor Cocktail (Roche Applied Sciences). Proteins from total cell lysates were resolved on SDS-PAGE (12% or 15%) and transferred to a nitrocellulose membrane. The membranes were blocked with PBS-T containing 5% nonfat dry milk for 2 h at room temperature and then probed with the indicated antibodies by incubation at 4°C overnight. Immune complexes were detected with HRP-conjugated secondary antibody and were visualized by ECL. The hippocampuses and stratums from rat brain were washed with PBS, dounced and then lysed with NP40 buffer; and western blotting was performed as described above.

Co-immunoprecipitation for detecting Beclin 1 and Bcl-2 interaction. Beclin 1/Bcl-2 co-immunoprecipitations were performed in morphine-treated SH-SY5Y cells lysed with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, and Protease Inhibitor Cocktail) on ice for 1 h. Immunoprecipitation of Bcl-2 was performed overnight at 4°C with the anti-Bcl-2 antibody (1:200 dilution). Immunoprecipitates were collected by incubating with 20 µl protein A-Agarose for 2 h at 4°C, followed by centrifugation for 1 min. The pellets were washed three times with Triton-X100 lysis buffer, and beads were boiled in loading buffer. Immunoprecipitates were subjected to 12% SDS-PAGE, and Beclin 1 and Bcl-2 were detected by western blot analysis.

Detection of cell death by PI staining. Cells were plated in six-well plates and treated with morphine. At the end of the experiment, cells were digested with Trypsin-EDTA solution, then collected by centrifugation and washed twice with ice-cold PBS, then cells were stained with 5 µl 50 µM PI. Flow cytometric analysis was performed to monitor the red fluorescence of DNA-bound PI (630 ± 22 nm). All data were analyzed with Cell Quest software (BD).

Morphine treatments. Rats were divided into four groups (4 rats in each group) and injected subcutaneously with saline or 10 mg/kg morphine hydrochloride for 9 days. The animals received 18 injections of saline (control) or morphine ("chronic" group), 17 injections of saline preceding an injection of morphine ("acute" group), or 17 injections of saline with an injection of 5 mg/kg naloxone (Sigma-Aldrich, USA) 30 min before the last injection of morphine ("acute + naloxone" group). Animals in

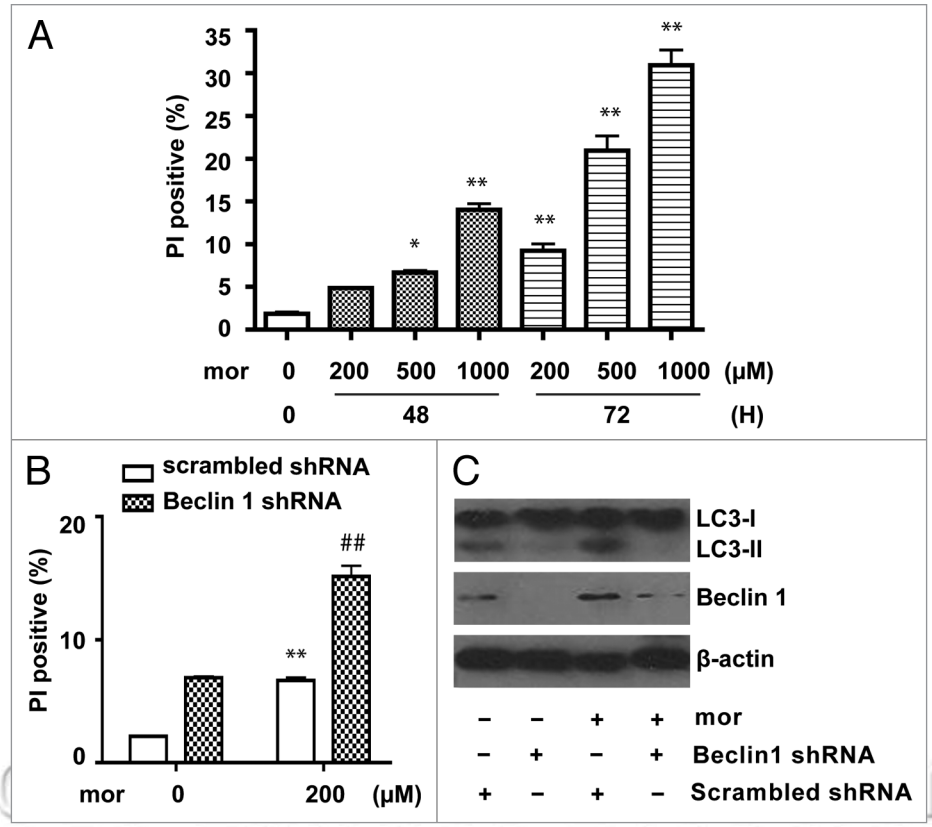

Figure 6. Morphine induces cell death, which is increased by autophagy inhibition. (A) SH-SY5Y cells were treated with 0, 200, 500, 1,000 μM morphine for 48, 72 h, then cells were harvested and stained with PI, detected by flow cytometry analysis. The amounts of PI positive cells were quantified. Results were shown as average ± SD for three separate experiments. *p < 0.05, **p < 0.01 compared with control. (B) *Beclin 1* shRNA or scrambled shRNA SH-SY5Y cells were treated with or without 200 μM morphine for 72 h, then cells were harvested and stained with PI, detected by flow cytometry analysis. The amounts of PI positive cells were quantified. Results were shown as average ± SD for three separate experiments. **p < 0.01 compared with control. ##p < 0.01 compared with scrambled shRNA SH-SY5Y. (C) *Beclin 1* shRNA or scrambled shRNA SH-SY5Y cells were treated with or without 200 μM morphine for 72 h, then cells were harvested and lysed with lysis buffer, Beclin 1, LC3-I and II levels were detected with anti-LC3 and anti-Beclin 1 antibodies.

the four groups above were sacrificed 2 h after the last injection, and the hippocampuses and striatums were isolated and prepared for western blot analysis. The experimental protocol and procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Statistical analysis. Data were analyzed as means ± SD. The data were evaluated statistically by the analysis of variance (ANOVA). Significance was determined as $p < 0.05$.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/ZhaoAUTO6-3-Sup.pdf

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