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Decreased mitochondrial DNA copy number in the hippocampus and peripheral blood during opiate addiction is mediated by autophagy and can be salvaged by melatonin

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Abbreviations: LC3, microtubule-associated protein 1 light chain 3; BAFA1, bafilomycin A_1; SSBP1, single-stranded DNA binding protein 1, mitochondrial; NRF1, nuclear respiratory factor 1; POLG, polymerase (DNA directed), gamma; POLRMT, polymerase (RNA) mitochondrial (DNA directed); ROS, reactive oxygen species; PC12, rat pheochromocytoma cell; i.p, intraperitoneally; DCFH-DA, 2′, 7′-dichlorofluorescin diacetate

Drug addiction is a chronic brain disease that is a serious social problem and causes enormous financial burden. Because mitochondrial abnormalities have been associated with opiate addiction, we examined the effect of morphine on mtDNA levels in rat and mouse models of addiction and in cultured cells. We found that mtDNA copy number was significantly reduced in the hippocampus and peripheral blood of morphine-addicted rats and mice compared with control animals. Concordantly, decreased mtDNA copy number and elevated mtDNA damage were observed in the peripheral blood from opiate-addicted patients, indicating detrimental effects of drug abuse and stress. In cultured rat pheochromocytoma (PC12) cells and mouse neurons, morphine treatment caused many mitochondrial defects, including a reduction in mtDNA copy number that was mediated by autophagy. Knockdown of the *Atg7* gene was able to counteract the loss of mtDNA copy number induced by morphine. The mitochondria-targeted antioxidant melatonin restored mtDNA content and neuronal outgrowth and prevented the increase in autophagy upon morphine treatment. In mice, coadministration of melatonin with morphine ameliorated morphine-induced behavioral sensitization, analgesic tolerance and mtDNA content reduction. During drug withdrawal in opiate-addicted patients and improvement of protracted abstinence syndrome, we observed an increase of serum melatonin level. Taken together, our study indicates that opioid addiction is associated with mtDNA copy number reduction and neurostructural remodeling. These effects appear to be mediated by autophagy and can be salvaged by melatonin.

Introduction

Drug addiction is a serious social problem and continues to cause an enormous financial burden for society. It is characterized by drug dependence, tolerance, sensitization and craving.[1] The available treatments remain inadequate for drug abusers, and addiction relapse rates vary depending on the substance being abused.[2] Therefore, an improved understanding of the biological changes underlying the process of drug addiction and abstinence will be important for developing more effective treatments.

Previous studies have demonstrated that metabolism and endocrine systems are actively involved in the process of long-lived behavioral abnormalities associated with addiction.[3,4] In addition, drug addiction is associated with many neurotoxic effects, which are often associated with oxidative stress.[5] Mitochondria play an important role in cellular metabolism and energy production, and also act as a primary source of reactive oxygen species (ROS) production that contributes to cell damage and apoptosis.[6,7] Cytoplasmic and mitochondrial enzymes have been identified as potential markers associated with drug dependence.[8] Chronic morphine treatment also results in mitochondria-dependent apoptosis[9] and mitochondrial dysfunction.[10] Mitochondrial function is strongly associated with the amount and quantity of mtDNA.[11] A high level of ROS production causes oxidative damage of mtDNA, which ultimately leads to a wide array of multisystem disorders, particularly affecting the energy-dependent

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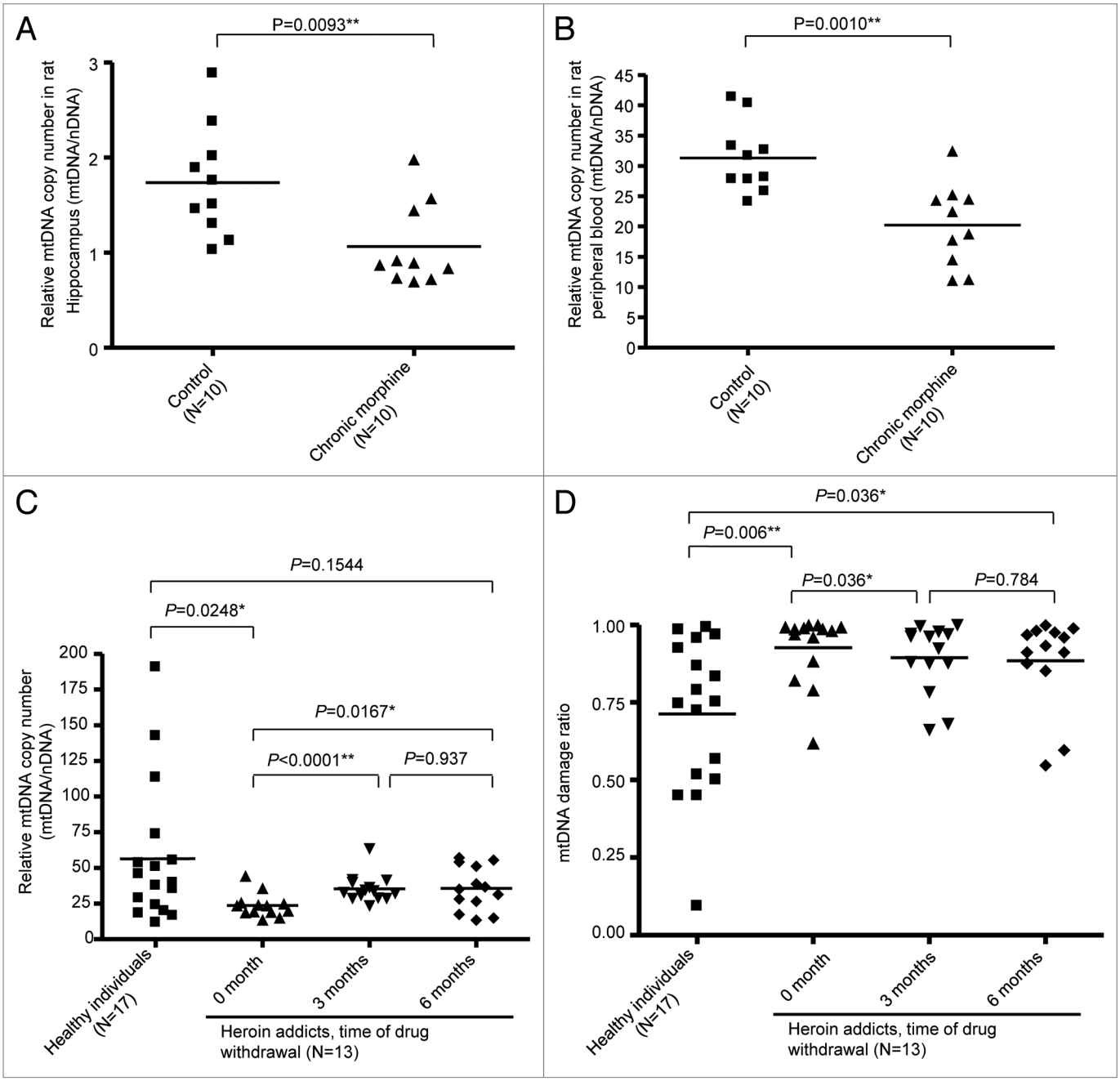


Figure 1. Alteration of mtDNA in rats with chronic morphine treatment and heroin-addicted patients. mtDNA copy number was significantly decreased in the hippocampus (**A**) and peripheral blood (**B**) of rats with chronic morphine treatment compared with controls. Heroin-addicted patients had lower mtDNA copy numbers (**C**) and a higher level of mtDNA damage (**D**) than those of normal subjects. *p < 0.05; **p < 0.01.

central nervous system.[12] Mitochondria are actively involved in regulation of synaptic plasticity,[13] and their dysfunction may underlie neurological changes in addiction. Nevertheless, the mechanisms that underlie potential mtDNA alterations observed in drug-addicted individuals and neuroplasticity have not been fully elucidated.

ROS, DNA damage and mitochondrial dysfunction can trigger autophagy, which is a tightly regulated pathway involving the lysosomal degradation of cytoplasmic organelles.[14,15] Neurons have highly dynamic cellular processes and are particularly vulnerable to oxidative stress, which leads to more autophagic activity.[16] Therefore, maintaining a balanced level of autophagy is critical for cell quality control and neuronal health. Previous studies have implicated both autophagy and mitochondrial quality control in regulation of neurite length and function in neuroplasticity.[17] We hypothesized that dysfunctional mitochondria induced by opiate-mediated neurotoxicity could be degraded by autophagy and eventually lead to neuronal pathological remodeling and plasticity. Here we provide direct evidence for a critical role of autophagy in regulating mtDNA

content and neurostructural remodeling during opioid addiction. Moreover, these effects can be rescued by melatonin.

Results

mtDNA copy number is decreased in the hippocampus and peripheral blood of rats upon chronic morphine treatment, and in the peripheral blood of opiate-addicted patients. We initially investigated if there is an association between mtDNA content and drug addiction by measuring mtDNA copy number in the hippocampus of rats with and without chronic morphine treatment. We observed a significant decrease of mtDNA copy number in the hippocampus tissue in rats receiving chronic morphine treatment compared with the controls ($p = 0.0093$) (**Fig. 1A**). Similar patterns were observed for the peripheral blood ($p = 0.0010$) (**Fig. 1B**), but not for the cortex, heart and liver (**Fig. S1**). Consistent with the observations in rat, heroin addicts had a lower level of mtDNA copy number in the peripheral blood ($p = 0.0248$) (**Fig. 1C**), and a higher level of mtDNA damage compared with healthy individuals ($p = 0.006$) (**Fig. 1D**). During the rehabilitation of drug abusers after drug detoxification for 3 mo, we observed a significant increase of mtDNA content ($p < 0.0001$) (**Fig. 1C**) and a decrease of mtDNA damage ($p = 0.036$) (**Fig. 1D**) compared with those at the initiation of drug withdrawal. Despite amelioration of mtDNA content and damage with time, these changes at both 3 and 6 mo were not restored to the levels similar to that of normal individuals. These results suggest that mtDNA alterations accompany drug addiction.

Morphine causes mitochondrial dysfunction and increased autophagy, leading to mtDNA copy number reduction. We performed experiments in morphine-treated rat pheochromocytoma (PC12) cells to extend the findings in rats and addicted patients. In accordance with the above results, mtDNA copy number was decreased in cells after morphine treatment, with the most significant effect at 20 μM (**Fig. S2A**). Since intracellular ROS is regarded as the main cause of mtDNA damage and mitochondrial dysfunction,[18] we measured ROS level in PC12 cells with and without morphine treatment. We observed elevated ROS level in cells receiving morphine treatment in a dose-dependent manner (**Fig. S2B–S2E**). Along with the ROS increase, we observed a significant decrease in all three parameters of oxygen consumption, i.e., overall respiration, basal mitochondrial respiration and ATP-coupled respiration, in cells treated with morphine (**Fig. S2F**). Intriguingly, an elevation of mitochondrial mass was observed in morphine-treated PC12 cells (**Fig. S2G**). We speculated that the increased mitochondrial mass could be a manifestation of mitochondrial dysfunction, as the remaining (and assumed fewer) mitochondria may be larger due to structural disorganization in the presence of morphine.

To examine whether the decreased mtDNA copy number during addiction was caused by a reduced expression of genes controlling mtDNA replication, we quantified mRNA expression of *Ssbp1*, *Nrf1*, *Polg* and *Polrmt*,[19,20] which have been reported to be actively involved in mitochondrial transcription and replication. We found little or no difference in the mRNA expression of these genes in the hippocampus tissue from rats with and without chronic morphine treatment (**Fig. S3**). However, mRNA expression of these genes was substantially increased in morphine-treated C6 cells (**Fig. S4**). Whether this increased expression reflected potential compensatory mitochondrial biogenesis or stress from mitochondrial dysfunction remains to be further clarified. Nonetheless, the reduction of mtDNA copy number during addiction is unlikely to be caused by decreased mtDNA replication.

Autophagy can be induced by ROS and mitochondrial damage.[21] To examine whether the mtDNA copy number reduction is caused by autophagy, we transfected PC12 cells, to express microtubule-associated protein 1 light chain 3 (MAP1LC3, hereafter LC3), with the N-terminally tagged pEGFP-C1-LC3 vector, together with a pDsRed-mito vector, which encodes a red fluorescent protein fused to a mitochondrial targeting sequence. Under steady-state conditions, EGFP-LC3 was diffusely localized in the cytosol and nucleus. EGFP-LC3 puncta were observed upon morphine treatment and about half of the puncta overlapped with mitochondria (**Fig. 2A**), suggesting that morphine treatment induces the degradation of mitochondria by autophagy.[22] Further immunofluorescence assays showed that morphine also induced endogenous LC3 puncta (**Fig. S5**). Accordingly, we observed an increased level of LC3B-II (the cleaved, lipidated and antophagosome-associated form of MAP1LC3B/LC3B) (**Fig. 2B**) and a decreased expression of SQSTM1/p62 (**Fig. 2C**). Bafilomycin A_1 (BAFA1) is an inhibitor of the vacuolar (V)-type ATPase that results in blockage of autophagosome-lysosome fusion.[23] In the presence of BAFA1, PC12 cells had an increased level of LC3B-II compared with control cells with morphine treatment (**Fig. 2D**). Moreover, there were more ultrastructure and vacuolar components that are most likely to be caused by autophagic activation in morphine-treated cells (**Fig. 2E and F**). We also found that the mRNA expression levels of several autophagy-related genes including *Atg3*, *Atg5*, *Atg7* and *Atg12* were upregulated after morphine treatment in C6 cells (**Fig. S6**).

We further performed experiments using siRNAs that targeted the *Atg7* gene, which encodes a key protein for the formation of autophagosome.[24] Among the three siRNAs for the *Atg7* gene, siRNA-3 had the best inhibitory effect (**Fig. S7**) and we used this siRNA in the subsequent knockdown assays. Concordantly with our expectation, knockdown of the *Atg7* gene, which increased mtDNA copy number, abolished the morphine-mediated reduction in mtDNA copy number (**Fig. 2G**), thereby providing direct evidence that this effect of morphine is autophagy-dependent. Knockdown of other autophagy-related genes such as *Lc3* (yeast *Atg8* homolog) and *Park2* had similar effects (data not shown). It is worth noting that the *Atg7* gene knockdown increased the mtDNA copy number (**Fig. 2G**) and ROS level (**Fig. 2H**) in untreated C6 cells. Taken together, our results suggest that mitochondrial defects induced by morphine can trigger autophagy that subsequently degrades and eliminates dysfunctional mitochondria, and ultimately results in mtDNA copy number reduction.

Melatonin restores mitochondrial function and prevents mtDNA copy number loss. Melatonin is a mitochondria-targeted antioxidant playing important roles on eliminating ROS

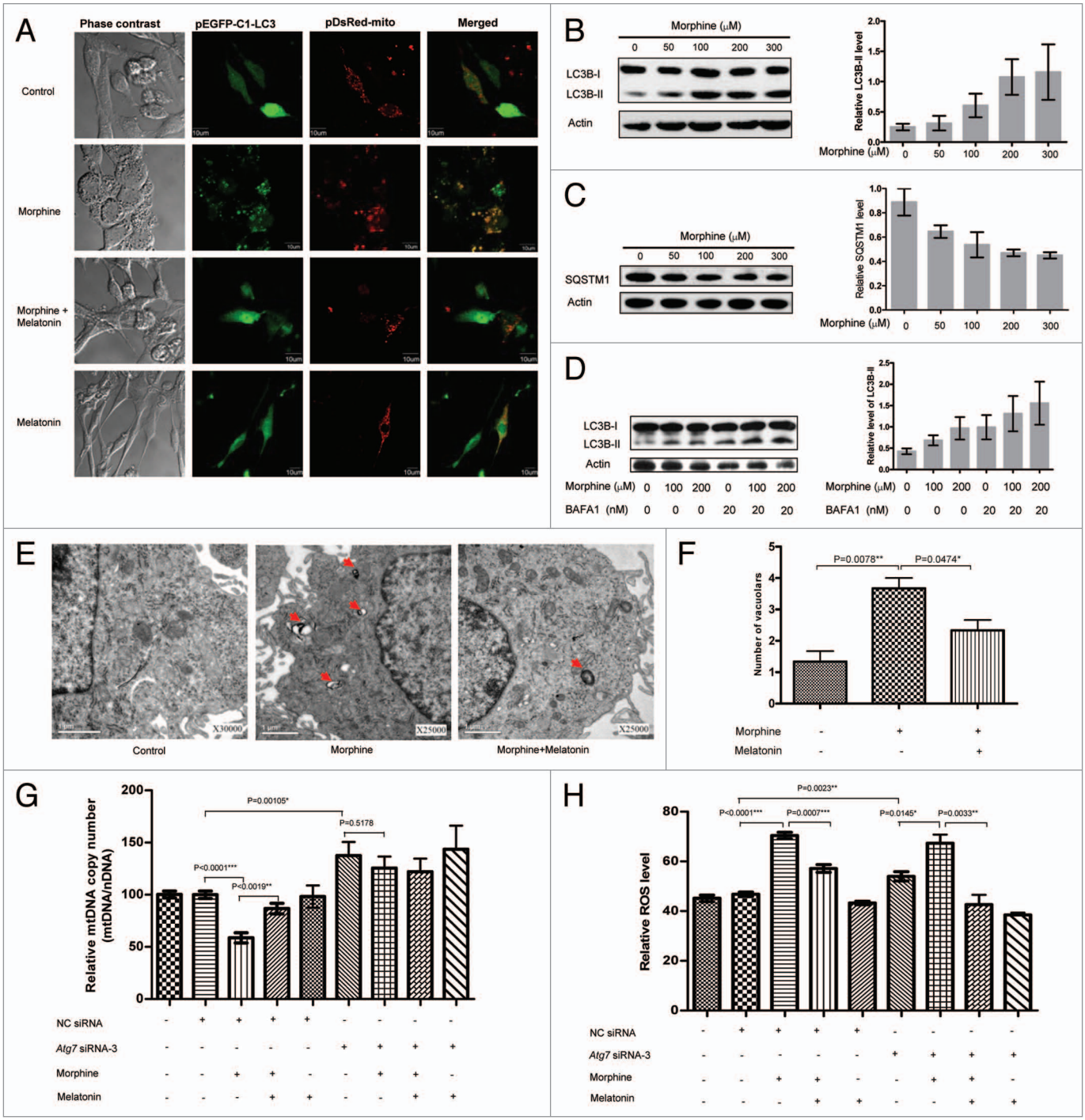


Figure 2. Morphine treatment induces autophagy in cultured cells and this effect can be rescued by melatonin. EGFP-LC3 puncta were induced by morphine (100 μM) treatment for 24 h and could be rescued by melatonin (100 μM) in PC12 cells (**A**). The increased level of LC3B-II (**B**) and decreased expression of SQSTM1 (**C**) showed a dose-dependent manner, and a treatment of 20 nM BAFA1 blocked the fusion of autophagosome and lysosome and led to accumulation of LC3B-II (**D**) in morphine-treated PC12 cells. A graphic representation of western blot data in each of three sections (**B–D**) is presented at the right side of a representative blot. Relative protein abundance was normalized to actin. Fewer secondary-lysosomes were observed under electron microscope in PC12 cells treated with both morphine (100 μM) and melatonin (100 μM) compared with morphine alone (100 μM) for 24 h (**E**). The number of the secondary-lysosomes was quantified in three cells of each group (**F**). Data are shown in (**A–F**) representative of four experiments. Knockdown of the *Atg7* gene increased mtDNA copy number (**G**) and ROS level (**H**) in C6 cells without morphine treatment. Morphine treatment had no significant effect on mtDNA copy number reduction (**G**), but still induced ROS elevation (**H**) in Atg7 knockdown cells. Data are representative of three experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

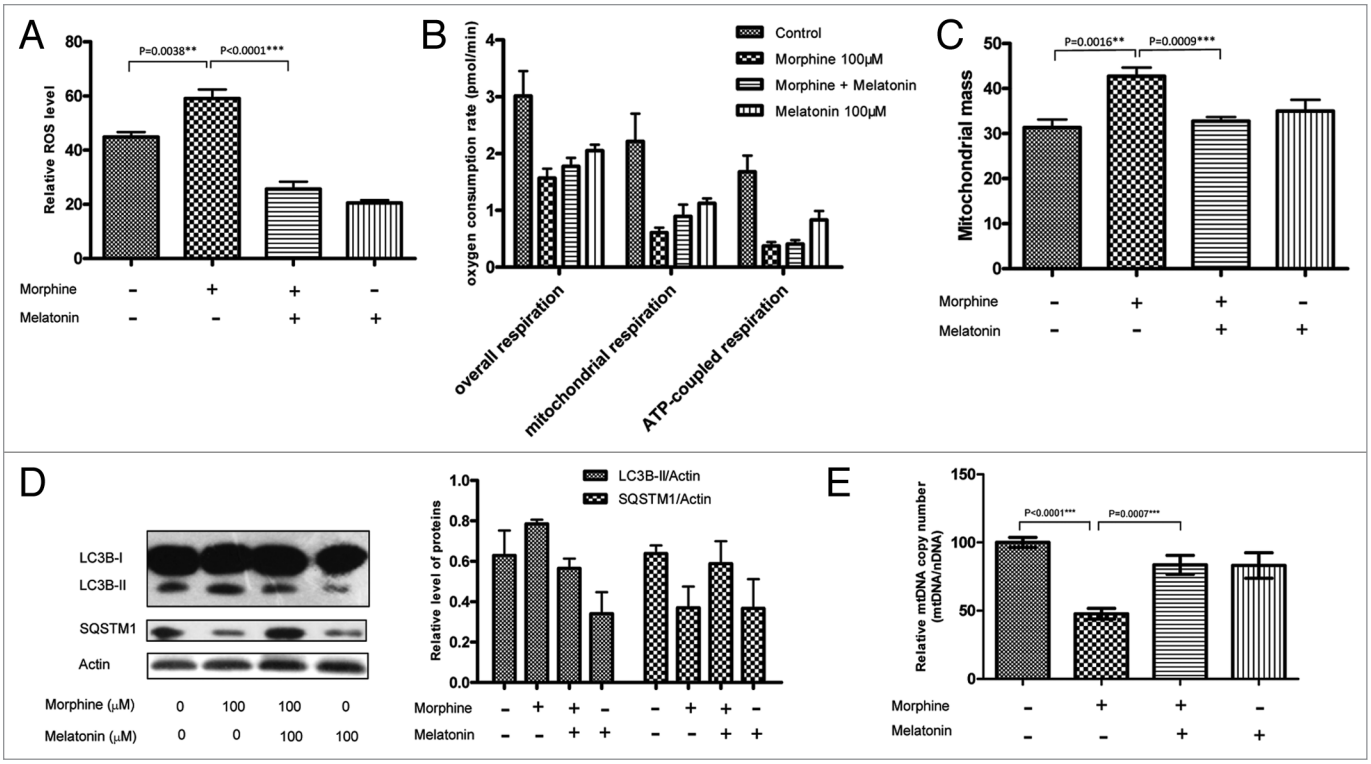


Figure 3. Morphine treatment induces mitochondrial dysfunction and autophagy in cultured cells and this effect can be rescued by melatonin. Pretreatment with melatonin (100 μM) restored the increased ROS level (**A**), decreased oxygen consumption rate (**B**), and elevated mitochondrial mass (**C**) induced by morphine (100 μM) in PC12 cells. The increased level of LC3B-II and decreased expression of SQSTM1 induced by morphine (100 μM) could be repeated in C6 cells and this effect could be suppressed by melatonin (100 μM) (**D**). Morphine treatment decreased mtDNA copy number and a pretreatment with melatonin restored this effect in C6 cells (**E**). Data are representative of four experiments. **p < 0.01; ***p < 0.001.

and repairing dysfunctional mitochondria.[25] It protects cells from methamphetamine-induced autophagy.[26] We sought to test whether melatonin could protect mitochondria against oxidative stress induced by morphine and further prevent autophagy. We found that melatonin blocked ROS elevation and ameliorated the impaired respiratory capacity induced by morphine in PC12 cells (**Fig. 3A and B**). It is worth noting that melatonin alone also had an inhibitory effect on respiration rate (**Fig. 3B**). Concordantly, melatonin salvaged the increased mitochondrial mass in response to morphine treatment, although melatonin itself slightly increased mitochondrial mass (**Fig. 3C**). Importantly, melatonin restored the levels of both LC3B-II and SQSTM1 in morphine treated C6 cells to a level similar to those of untreated cells (**Fig. 3D**) and reduced the number of the EGFP-LC3 puncta (**Fig. 2A**) triggered by morphine. In addition, melatonin also reduced the endogenous LC3 puncta induced by morphine (**Fig. S5**). Electron microscopy analysis confirmed that melatonin decreased the number of secondary lysosomes in morphine-treated cells (**Fig. 2E and F**), suggesting that melatonin treatment reduces autophagy, with the consequence of mtDNA recovery (**Fig. 3E**). Quantification of mRNA expression levels of the autophagy-related genes showed that melatonin was able to counteract the increase of *Atg3*, *Atg5*, *Atg7* and *Atg12* mRNA induced by morphine (**Fig. S8**).

Melatonin restores morphological changes of neurons induced by morphine. The morphological structure of a neuron is closely related to its function, and mitochondria are actively involved in maintaining the morphology and plasticity of neurons.[27] Dysfunctional mitochondria in neurons are degraded in the soma via autophagy.[28] Consistent with this notion, we observed the aggregation of LC3B in the soma of cultured mouse cortical neurons upon morphine treatment (**Fig. 4A**), indicating the degradation of dysfunctional mitochondria via autophagy. Melatonin restored LC3B redistribution, decreased LC3B-II level and prevented autophagic activation in morphine-treated neurons (**Fig. 4A and B**). We stained mouse primary cortical neurons for TUBB3/β-III tubulin. As expected, morphine treatment (100 μM for 24 h) reduced the length of TUBB3-positive axons and dendrites compared with those without treatment, and the shortened neurites induced by morphine were rescued by melatonin (**Fig. 4C**). Intriguingly, melatonin alone enhanced the length of TUBB3-positive axons and dendrites. These results indicate an active role of melatonin in regulating neuromorphological changes and counteracting the deleterious effects of morphine.

Melatonin rescues behavioral sensitization and analgesic tolerance in mice with chronic morphine treatment. To further discern whether melatonin also salvaged the morphine effects in vivo, we employed mouse models: repeated morphine treatment induced behavioral sensitization and analgesic tolerance. Behavioral sensitization is characterized by a progressive increase of locomotor following repeated drug exposure.[29] Analgesia was

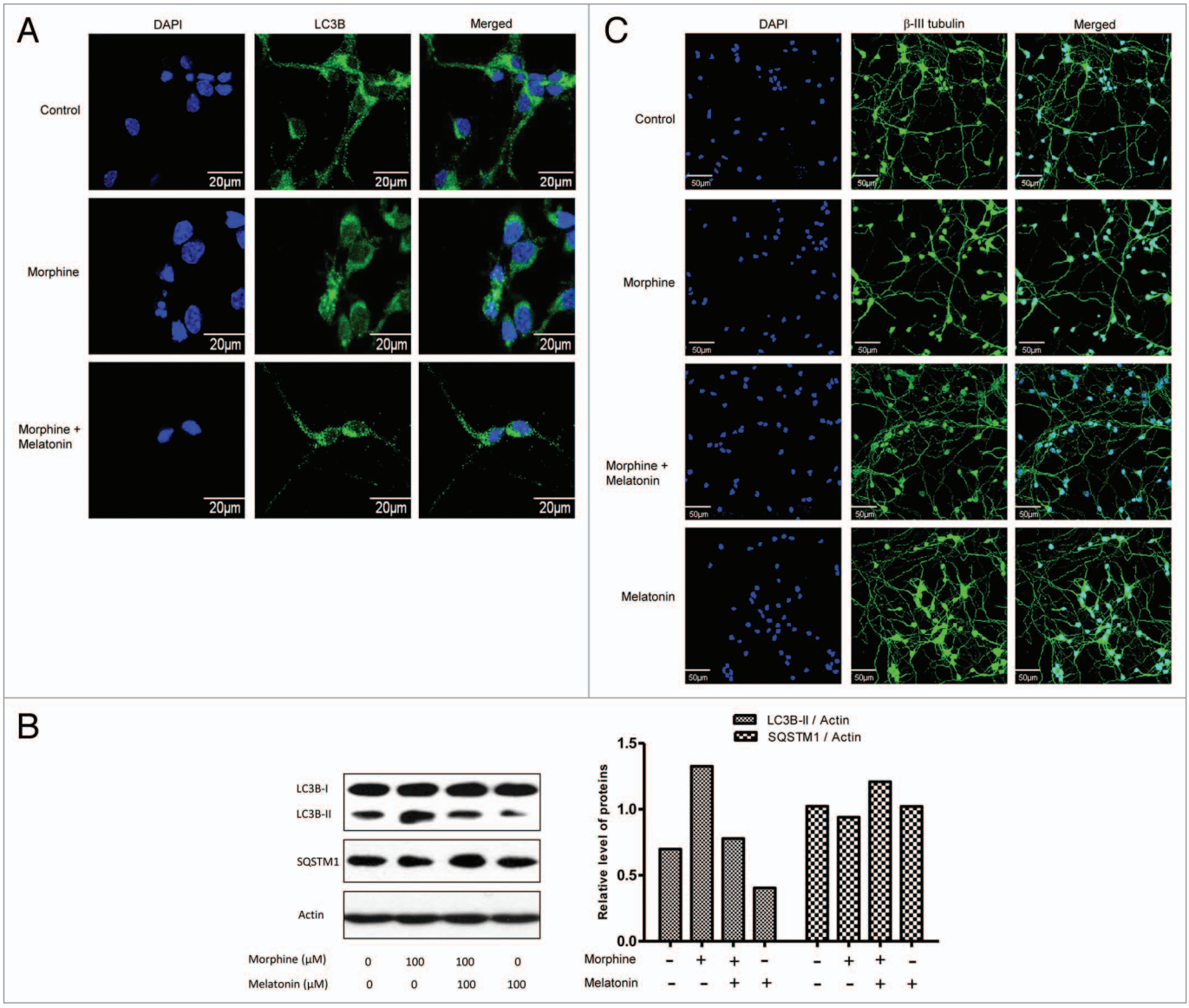
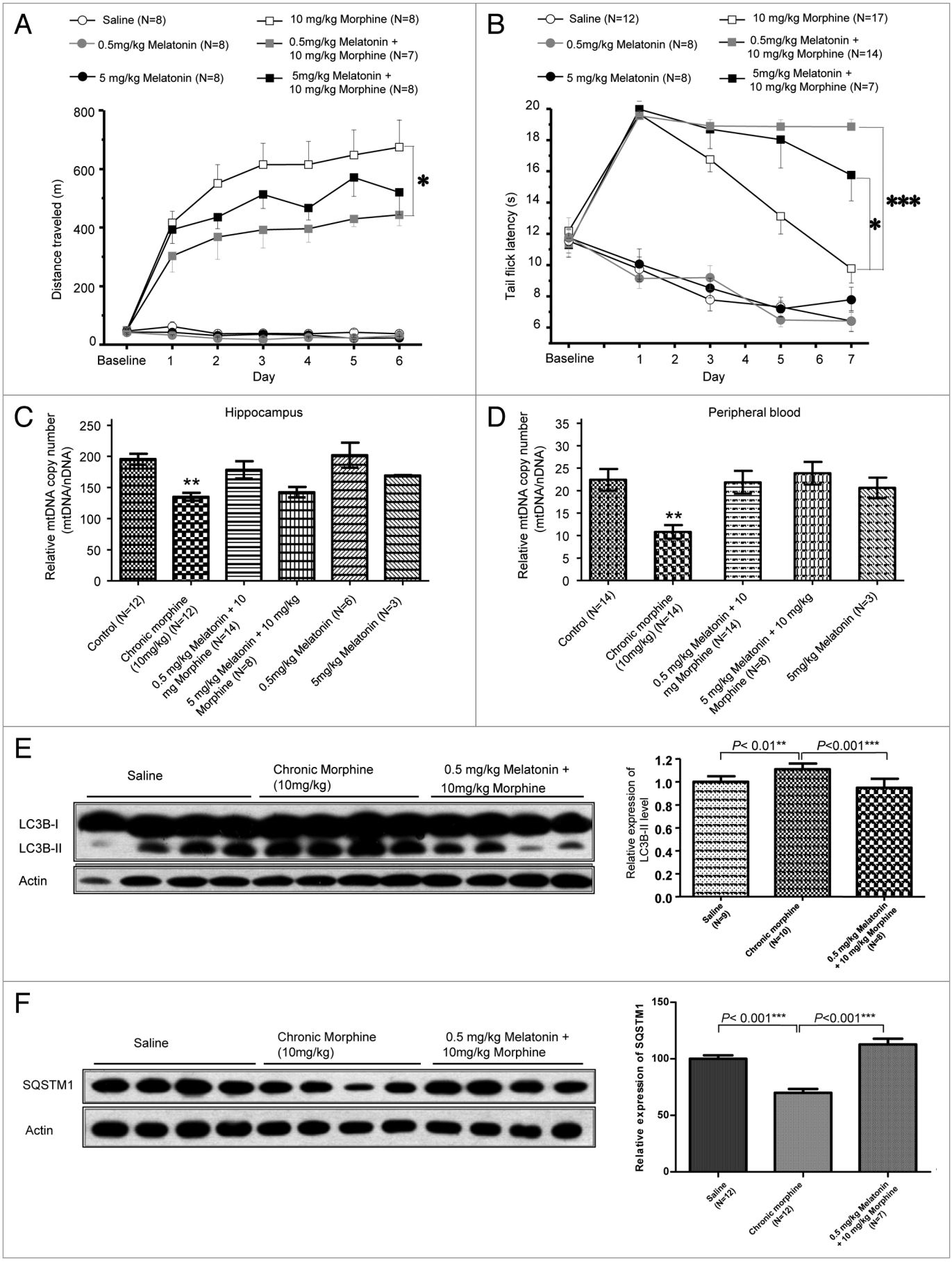


Figure 4. Effects of melatonin on primary neuron dendritic morphogenesis. Mouse primary cortical neurons were cultured in neurobasal medium with 2% B27. Neurons were pretreated with melatonin (100 μM) for 1 h, followed by treatment with or without 100 μM morphine. Upon morphine treatment, LC3B-II aggregated in neuron soma and this defect was restored by a pretreatment with melatonin (**A**). Melatonin treatment reduced the level of LC3B-II induced by morphine and slightly increased SQSTM1 expression (**B**). The appearance of shortened neurites (stained by TUBB3 and viewed under a fluorescent microscopy), induced by morphine was rescued by melatonin (**C**). Data are representative of two experiments.

assessed using the radiant heat tail-flick latency 60 min after morphine injection. The locomotor response was significantly enhanced in mice subjected to 10 mg/kg morphine (s.c.) once per day for 6 d compared with the saline controls. Pretreatment with 0.5 mg/kg melatonin (i.p.) in mice significantly rescued the increased locomotor activity induced by morphine. The salvaging effect of melatonin was dose-dependent, with a stronger effect at lower concentration (**Fig. 5A**). Similarly, melatonin blocked

Figure 5 (See opposite page). Melatonin treatment counteracts the effects of morphine on behavioral sensitization and analgesic tolerance in mouse models. All values are expressed as mean ± SEM. There was a steady increase of locomotor activity in the mean behavioral scores upon repeated morphine injections. Pretreatment with melatonin (0.5 mg/kg and 5 mg/kg) significantly reduced behavioral sensitization compared with the morphine group (**A**). Mice with chronic morphine administration (10 mg/kg once daily) showed an analgesic tolerance as mirrored by the gradually decreased tail-flick latency response. Cotreatment with melatonin and morphine reversed morphine-induced analgesic tolerance (**B**). There is a decrease of mtDNA copy number in the hippocampus (**C**) and peripheral blood (**D**) of the chronic morphine-treated mice, while melatonin countered morphine effects on mtDNA copy number reduction. ** $p < 0.01$, compared with control group. Injection of 0.5 mg/kg melatonin restored the elevated LC3B-II level (**E**) and the decreased expression of SQSTM1 (**F**) induced by morphine in the hippocampus tissues. The relative LC3B-II levels (**E**) and SQSTM1 expression (**F**) between the control group and morphine group and between morphine group and morphine plus melatonin group were statistically significant. **$p < 0.01$; ***$p < 0.001$.



analgesic tolerance induced by morphine (**Fig. 5B**). Consistent with the finding in rats, we observed a significant decrease of mtDNA copy number in the hippocampus and peripheral blood of mice with chronic morphine treatment, and this effect was rescued by melatonin (**Fig. 5C and D**). Western blot analysis showed that the elevated LC3B-II and decreased SQSTM1 expression levels in mouse hippocampus induced by chronic morphine treatment were both restored to control levels by 0.5 mg/kg melatonin pretreatment (**Fig. 5E and F**). Ultrastructure analysis showed that morphine injection induced the autophagasome, an effect that was reduced by melatonin administration in mice (**Fig. S9**). These results suggest that melatonin salvages the mtDNA copy number loss through inhibition of morphine-induced autophagy.

Heroin-addicted patients undergoing withdrawal show increased serum melatonin levels. During rehabilitation, heroin-addicted patients showed the recovery of mtDNA copy number and reduction of mtDNA damage (**Fig. 1**). Because our results showed the similar benefit by melatonin treatment (**Fig. 3**), we wondered whether drug rehabilitation might, at least in part, operate via melatonin. Compared with healthy individuals, drug abusers had significantly lower levels of melatonin ($p = 0.0014$) (**Fig. 6A**). After drug withdrawal for 3 and 6 mo, we observed a significant increase of serum melatonin levels ($p < 0.005$), consistent with the improvement of mtDNA copy number with time. However, the serum melatonin levels in the patients did not fully recover to that of healthy individuals (**Fig. 6A**). Collectively, these results demonstrate that alteration of mtDNA content via autophagy might be involved in drug addiction and that melatonin has protective effects on this process (**Fig. 6B**).

Discussion

Mitochondria are enriched in growth cones, axon terminals and dendrites of neurons, and play important roles in regulating neurite outgrowth and synaptic plasticity in the mature nervous system.[28,30] Studies of hippocampal neurons have shown that mitochondria are involved in the morphogenesis and plasticity of spines and synapses.[31] Mitochondrial dysfunction and abnormality are hallmarks of many brain disorders.[30] In particular, changes in mitochondrial content via autophagy play important roles in regulation of neuronal plasticity and pathological remodeling.[28,30] In this study, we tested whether alteration of mtDNA copy number might be a hallmark of addiction. Indeed, we observed lower mtDNA copy numbers in the hippocampus and peripheral blood of rats chronically exposed to morphine. This finding was validated in mice and heroin-addicted patients (**Fig. 1**; **Fig. 5**), suggesting that it is a common phenomenon during addiction.

To elucidate the potential mechanism of the decreased mtDNA copy number in response to chronic morphine treatment, we treated cultured PC12 cells with morphine and observed increased ROS, diminished oxygen consumption, decreased mtDNA copy number and elevated mitochondrial mass (**Fig. S2**). These results suggest that mitochondrial dysfunction and mtDNA changes are actively involved in the process of drug addiction. Previous studies have shown that apoptosis is involved in neuronal dysfunction induced by opiates in mouse cortex, hippocampus, neurons and PC12 cells,[10,32] but the exact molecular mechanisms of neurotoxicity induced by opiate drugs remains largely unclear. The neurotoxic effects are often associated with excessive oxidative stress, which is detrimental to lipids, proteins and DNA of the cell, especially to the "naked" mtDNA that lacks histone protection.[33] Autophagy, which can be induced by increased ROS, has the capability to remove damaged proteins and organelles and thus maintains cellular homeostasis, while severe autophagy leads to neurotoxicity and autophagic cell death.[34] Because we observed increased ROS in PC12 cells with morphine treatment and elevated mtDNA damage in heroin-addicted patients, we speculate that these defects would trigger autophagy that eliminated dysfunctional mitochondria and led to a reduction of mtDNA copy number.

Consistent with a recent study that demonstrates the involvement of BECN1 and ATG5-dependent autophagy in human neuroblastoma SH-SY5Y cells and in rat hippocampus in response to morphine treatment,[35] we found that morphine treatment of PC12 cells induced EGFP-LC3 puncta (**Fig. 2**) that partially overlapped with the distribution of mitochondria. This increase in autophagy was further validated by western blot analysis and electron microscopy. Further experimental assay with knockdown of *Atg7* (**Fig. 2**) and other autophagy-related genes (data not shown) in C6 cells showed that morphine no longer caused a significant reduction of mtDNA copy number. All these observations suggested that autophagy could eliminate defective mitochondria and result in decreased mtDNA copy number. Because mitochondrial dynamics affects the morphology and structure of neurons, we validated our findings from PC12 cells using mouse primary cortical neurons (**Fig. 4**). Remarkably, morphine treatment reduced the length of axons and dendrites in neurons and increased autophagy, indicating that autophagy contributed to neuron structure remodeling. Taking all these findings together, it is clear that morphine-treated cells and animals show reduced mtDNA copy number that is associated with increased autophagy. Following this thread, reduction of mtDNA copy number may be a biomarker for opiate addiction, and prevention of the reduction of mtDNA copy number may be beneficial to the clinical treatment of addiction.

During protracted drug withdrawal, opiate addicts show disruptions in the circadian rhythms of hormones, which may result in long-lasting neurobiological changes that increase the likelihood of relapse.[36] Melatonin is the main secretory product of the pineal gland that regulates circadian rhythms and has well-known antioxidant properties.[37] Melatonin inhibits autophagy through a redox-mediated mechanism,[38] although some studies claim that melatonin even induces autophagy.[39] Therefore, we tested whether melatonin relieved the deleterious effects of opiate during addiction. Consistent with previous studies,[40] we found that melatonin was able to rescue the increased ROS and mitochondrial dysfunction induced by morphine in cultured cells (**Fig. 3**). Most importantly, melatonin rescued morphine-induced autophagy and ameliorated decreased mtDNA copy number in response to morphine treatment in cultured cells and neurons (**Figs. 2–4**) and in a mouse model of morphine addiction (**Fig. 5**).

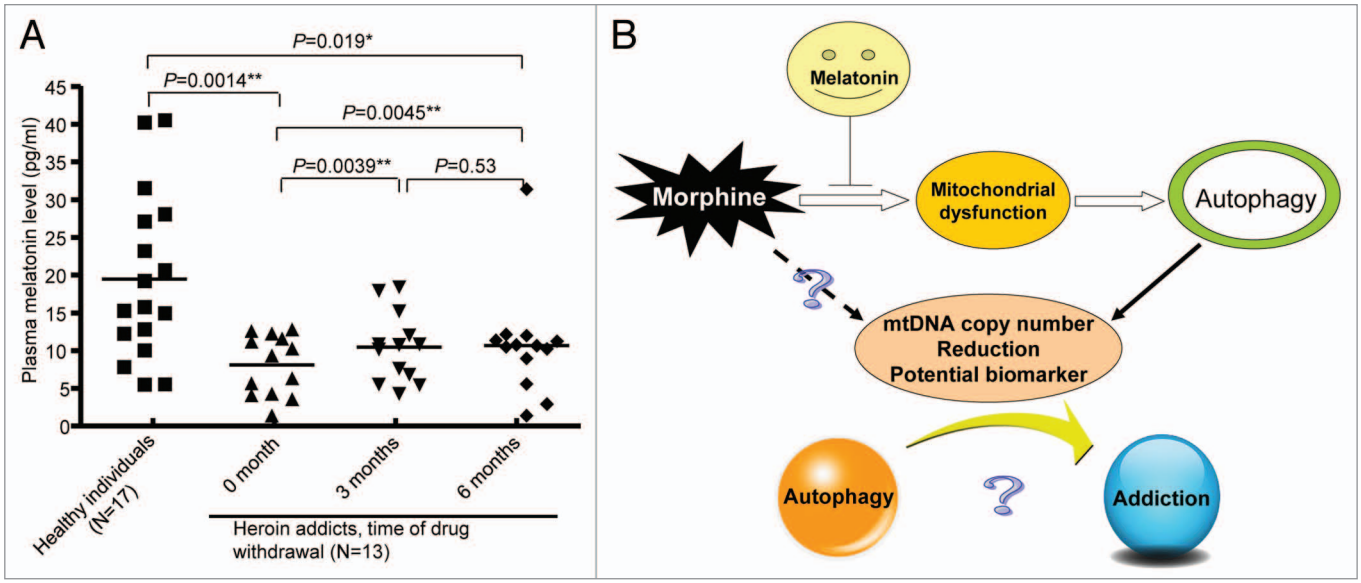


Figure 6. Serum melatonin in heroin-addicted patients after drug withdrawal (**A**) and a schematic diagram of potential relationship between morphine, autophagy and addiction (**B**). Serum melatonin was measured in heroin-addicted patients at time of withdrawal for 0, 3 and 6 mo. *p < 0.05; **p < 0.01.

All these results suggest that melatonin has beneficial effects on mitochondrial functions during drug addiction. In particular, we found that pretreatment with melatonin significantly prevented morphine-induced behavioral sensitization and analgesic tolerance, and these protective effects were dependent on the melatonin concentration (**Fig. 5**). Melatonin counteracted the autophagy effect induced by morphine in the hippocampus tissues from these morphine-addicted animals, which would salvage mtDNA copy number loss (**Fig. 5**). These findings are consistent with previous observations on the beneficial effects of melatonin[41-44] and provide the molecular underpinning of this biological process. It is worth noting that melatonin itself increased the levels of LC3B-II in mouse hippocampus tissues, but it decreased the levels of LC3B-II in C6 cells (**Fig. S10**). The exact reason for this conflicting effect under different conditions remains unknown.

Our findings from morphine-addicted animals have important implications for treatment of opiate-addicted patients. Heroin-addicted patients have a significantly lower level of serum melatonin and mtDNA copy number compared with healthy individuals (**Fig. 1**; **Fig. 6**). After drug withdrawal, we observed a corresponding increase of plasma melatonin and mtDNA copy number and a decrease of mtDNA damage, although the levels did not fully restore to that of normal individuals. Moreover, the first three months after detoxification is the critical stage for the recovery of plasma melatonin level and amelioration of mtDNA in blood cells. These findings offer direct evidence for the importance of monitoring mtDNA copy number in opiate-addicted patients after drug withdrawal. Prescription of melatonin, which provides a compensatory and protective role in eliminating ROS and suppressing autophagy, would help the addicted patients to recovery from opioid withdrawal.

In summary, we found that opiate addiction was tightly associated with decreased mtDNA copy number in the hippocampus and peripheral blood. This was due to increased autophagy and could be salvaged by melatonin. The detrimental effects of morphine treatment on cultured PC12 cells, primary neurons and animal models were also counteracted by melatonin. Furthermore, there was a corresponding increase of serum melatonin and mtDNA copy number in heroin-addicted patients following the duration of drug withdrawal, the longer the better. These findings may have important implications for the treatment of opiate-addicted patients. Meanwhile, our study raises further questions. What is the exact biological significance of mtDNA copy number loss during addiction? A recent study shows that the mtDNA escaped from autophagy-mediated degradation causes inflammation and heart failure.[45] Does mtDNA loss during opiate addiction has similar effects? Which genes and key pathways bridge morphine, mitochondria, autophagy, and synaptic plasticity? We showed that knockdown of the *Atg7* gene ameliorated the morphine-induced mtDNA copy number loss, but the exact mechanism still remains unknown. Further studies are essential to answer these intriguing and important questions.

Materials and Methods

A detailed methods section is available in the Supplemental Materials.

Animal models and patients. Male Sprague Dawley rats and male Kunming mice were obtained from animal core facility of the Kunming Medical College. Rats were given chronic morphine treatment following a previously published procedure[46,47] and 5 tissues (including hippocampus, prefrontal cortex, liver, heart and blood) were collected. We created mouse models for behavioral sensitization and analgesic tolerance by morphine injection. Thirteen female heroin addicts (mean age 31.7 ± 2.4 y) at time of withdrawal were recruited from the Kunming Rehabilitation Center on Drug Dependence in Yunnan and were followed up

for 6 mo. Seventeen drug-free healthy volunteers (mean age 30 ± 1.7 y), without any history of medical, neurological or psychiatric diseases, were used as the control samples. These individuals were first reported in our previous study.[48] Serum samples were collected and stored at -80°C. All the subjects provided informed consent prior to the study. The institutional review board of Kunming Institute of Zoology approved this study.

Measurement of mtDNA copy number and mtDNA damage. Genomic DNA was extracted by the Genomic DNA Miniprep Kit (Axygen, AP-MN-BL-GDNA-250). mtDNA copy number was measured by using real-time quantitative PCR (qPCR) and was normalized to the *Hbb* (β-globin) gene (**Tables S1 and S2**). qPCR was performed on the platform of iQ2 system (BioRad Laboratories) with SYBR® Premix Ex Taq™ II kit (TaKaRa, DRR081A). We employed a previously described method[49] to quantify mtDNA damage ratio (**Table S1**).

RNA extraction, RT-qPCR assay and western blot. Total RNA was isolated from tissues and cell lines using TRIZOL (Invitrogen, 15596-018) and cDNA was synthesized by using the M-MLV Reverse Transcriptase (Promega, M170A) according to the manufacturer's instructions. The relative mRNA expression levels of mitochondrial transcription and replication-related genes, and autophagy-related genes (**Table S3**) were quantified by RT-qPCR, with normalization to the *Actb*/β-*actin* gene. Western blots for the target proteins were performed using standard methods. Cell lysates were prepared using protein lysis buffer (Beyotime, P0013). Protein concentration was determined using a protein assay reagent (Bio-Rad, 500-0006). The membrane was incubated with primary antibodies against LC3B (Cell Signaling Technology, Inc., 3868, 1:1000, overnight at 4°C), SQSTM1/p62 (Merck Milipore, MABC32, 1:1000, overnight at 4°C) and ACTB (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, TA-09, 1:10,000, overnight at 4°C), followed by the peroxidase-conjugated anti-mouse (KPL, 474-1806, 1:5000) or anti-rabbit (KPL, 474-1506, 1:5000) IgG for 1 h at room temperature. The epitope was visualized using an ECL western blot detection kit (Millipore, WBKLS0500).

RNA interference. Three siRNAs of the *Atg7* gene and the negative control were obtained from RiboBio. siRNAs transfection was performed by using Lipofectamine ™ 2000 (Invitrogen, 11668-027) according to the manufacturer's instruction. The efficiency of siRNA to downregulate the expression of the *Atg7* mRNA was determined by RT-qPCR analysis.

Measurement of ROS, mitochondrial mass and oxygen consumption rate. We used DCFH-DA (2′, 7′-dichlorofluorescin diacetate; Sigma-Aldrich, D6883) staining to quantify cellular ROS and the MitoTracker Red (Molecular Probe, M22425) to detect the change of mitochondrial mass. Cellular oxygen consumption was measured by using a Clark-type oxygen sensor (Hansatech instruments, England).

Immunocytochemistry, confocal microscopy and electron microscopy assays. We discerned autophagy in PC12 cells, primary neurons and tissues using two approaches. First, cultured cells were cotransfected with N-terminally tagged pEGFP-C1-LC3 (a kind gift from Dr. Quan Chen) and pDsRed-Mito vectors (Clontech, 632421). Intact cells were imaged by using an Olympus FluoView™ 1000 confocal microscope (Olympus) after being treated with morphine and/or melatonin. For immunocytochemistry, cells were fixed and stained with antibodies against MAP1LC3B/LC3B (Cell Signaling Technology, 3868; 1:50) and TUBB3/β-III tubulin (Abcam, ab7751; 1:200). Immunoreactivity was detected with FITC-conjugated secondary antibody (KPL, 172-1506; 1:50) and nuclei were counterstained with DAPI (Roche, 10236276001). Subsequently, a transmission electron microscope (JEOL, JEM-1011) was used to identify autophagosomes.

Quantification of serum melatonin in heroin-addicted patients and normal controls. Serum melatonin concentration in normal individuals and heroin addicts at time of withdrawal, 3 and 6 mo after withdrawal were measured by enzyme-linked immunosorbent assay kit (IBL Company, RE54021). The procedure followed the basic principle for competitive immunoassays and the limit of detection was 1.6 pg/mL.

Statistical analysis. We used unpaired t-tests to quantify differences of mtDNA copy number in tissues from rats and mice with and without morphine treatment and the levels of serum melatonin, mtDNA copy number and damage in healthy individuals and heroin addicts. Paired t-tests were employed to quantify difference of serum melatonin and mtDNA alterations in patients at time of drug withdrawal for 0, 3 and 6 mo. Differences in animal behavioral sensitization and analgesic tolerance were determined by ANOVA, followed by LSD (Least Significant Difference) for post-hoc comparisons.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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Online Supplement

Methods

Reagents, chemicals and cells

The following antibodies and chemicals were used in this study: rabbit monoclonal anti-MAP1LC3B/LC3B (#3868; Cell Signaling Technology, Inc.), SQSTM1/p62 (Merck Milipore, MABC32), mouse monoclonal anti-ACTB/β-actin (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, TA-09), mouse monoclonal anti-TUBB3/β-III tubulin (Abcam, ab7751), FITC-conjugated anti-rabbit antibody (KPL, 172-1506). 2′, 7′-Dichlorofluorescin diacetate (DCFH-DA) (Sigma, D6883), Vitamin K3 (Sigma, M5625), melatonin (Sigma, M5250) and poly-L-ornithine (Sigma, P4957). Morphine Hydrochloride for injection was bought from Shenyang Pharmaceutical Co. Ltd. (State Drug Approval Document Number: H21022436, Shenyang, China). BAFA1 was purchased from Invivogen (tlrl-baf). DAPI was bought from Roche Diagnostics (10236276001).

Rat pheochromocytoma PC12 cells and glioma C6 cells were obtained from Kunming Cell Bank, Kunming Institute of Zoology. Cells were maintained in DMEM medium (Gibco-BRL, 11965-092) with 10% fetal bovine serum (Gibco-BRL, 10099-141) at 37^{o}C in a humidified atmosphere incubator with 5% CO_2.

Establishment of a chronic morphine-addicted model in rats

Experiments were performed on male Sprague Dawley rats weighing 200 to 250 g. Animals were group-housed under a 12 h light/dark cycle with free access to water and food. The animal care and experimental protocol was approved by the Kunming Institute of Zoology, Chinese Academy of Sciences. Rats were chronically treated with morphine hydrochloride (10 mg/kg, subcutaneously [s.c.]) twice per day at 12 h intervals for 12 d, following the previously described procedures.[1-4] After that, the rats were sacrificed and 5 tissues (including hippocampus, prefrontal cortex, liver, heart, and blood) were collected.

Patients

A total of 13 female heroin addicts, with the mean age of 31.7±2.4 years, at time of drug withdrawal were recruited from the Kunming Rehabilitation Center on Drug Dependence in Yunnan, China and were followed up for 6 months. Blood samples were collected from each patient in the morning between 7:00 am and 9:00 am at time of 0, 3, and 6 months. 17 drug-free healthy volunteers, aged 30±1.7 years, without any history of medical, neurological or psychiatric diseases, provided the control samples representative of the general population. Serum was separated from blood by centrifuging at 2000 g for 10 min and was frozen at -80^{o}C until further processing. All the subjects provided informed consent prior to the study. The institutional review board of Kunming Institute of Zoology approved this study.

Measurement of mtDNA copy number and mtDNA damage

Genomic DNA was extracted by using the AxyPrep™ Blood Genomic DNA Miniprep Kit (Axygen, AP-MN-BL-GDNA-250). mtDNA copy number was measured using the real-time quantitative PCR (qPCR) and the relative standard curve method. mtDNA content was normalized to the single-copy nuclear *Hbb* (β-globin) gene. For mtDNA damage ratio examination, we employed the methods of Rothfuss and coworkers.[5] The primer pairs for measuring mtDNA copy number and damage are listed in Tables S1 and S2. qPCR was performed on the platform of iQ2 system (BioRad Laboratories, Hercules, CA, USA) with

SYBR® Premix Ex Taq™ II kit (TaKaRa, DRR081A).

RNA extraction and RT-qPCR assay

Total RNA was isolated from rat hippocampus tissues and glioma C6 cells using TRIZOL (Invitrogen, 15596-018). 1 μg of total RNA was used to synthesize single-strand cDNA using the M-MLV Reverse Transcriptase (Promega, M170A) in a final volume of 25 μL reaction mixture according to the manufacture's instructions. The relative mRNA expression levels of *Ssbp1* (mitochondrial single stranded DNA binding protein 1), *Nrf1* (nuclear respiratory factor 1), *Polg* (polymerase (DNA directed), gamma) and *Polrmt* (polymerase (RNA) mitochondrial (DNA directed)) were normalized by the housekeeping gene *Actb*/*β-actin*. The primer pairs used for these mitochondrial transcription and replication-related genes, and autophagy-related genes were listed in Table S3.

RNA interference

Three siRNAs of the *Atg7* gene and negative control siRNA (NC siRNA) were obtained from RiboBio (Guangzhou, China). C6 cells were cultured in DMEM medium with 10% fetal bovine serum at 37^{o}C in 5% CO_2. In brief, cells (1×10^5 per well) were seeded in 12-well plates to grow to 50% confluence. Before transfection, culture medium was removed and washed once with Opti-MEM medium (Gibco-BRL, 31985-070). *Atg7* siRNA or NC siRNA was dissolved in Opti-MEM medium, then mixed with 1 μL Lipofectamine ^{TM} 2000 (Invitrogen, 11668-027) to achieve a final volume of 100 μL. The siRNA-Lipofectamine mixture was incubated at room temperature for 20 min before adding to each well, together with an additional 400 μL Opti-MEM medium. After incubation for 6 h in the CO_2 incubator, the medium was removed and 1 mL fresh medium was added to each well for growth. We optimized the siRNA concentration for transfection and the time for cell harvest by testing five different concentrations of siRNA (6.25 nM, 12.5 nM, 25 nM, 50 nM, and 100 nM) and three harvest time points (24, 36 and 48 h). The efficiency of siRNA to downregulate the expression of the *Atg7* mRNA was determined by RT-qPCR analysis.

Measurement of reactive oxygen species in PC12 cells

PC12 cells were cultured in DMEM supplemented with 10% fetal bovine Serum in a humidified atmosphere incubator with 5% CO_2 at 37°C. After 24 h of morphine treatment, cells were harvested and washed once with PBS. 2 μM DCFH-DA was used to examine the intracellular reactive oxygen species (ROS) level. Cells were incubated with the dye at 37°C for 20 min, then washed twice with PBS, resuspended in PBS and kept on ice for an immediate detection on the FACScan (Becton Dickinson, USA).

Measurement of mitochondrial mass level and oxygen consumption rate in PC12 cells

We used MitoTracker Red (Molecular Probe, M22425) to detect the change of mitochondrial mass. Cells were trypsinized using 0.25% trypsin-EDTA, pelleted and resuspended in prewarmed medium containing 100 nM MitoTracker Red at 37°C for 30 min, then were washed with prewarmed medium and PBS. The mitochondrial mass of the stained cells were examined by using the FACScan at 644 nm for excitation.

Cellular oxygen consumption was measured using a Clark-type oxygen sensor (Oxytherm; Hansatech Instruments Ltd, Norfolk England) at 25^{o}C, following our recent approach.[6] Briefly, 2×10^6 cells were analyzed in culture medium with and without chemicals. After having recorded the respiration of cells for 5 min, ATP synthase inhibitor oligomycin A (Sigma, 75351) (1 μM) was added into the chamber

to collect the ATP-coupled respiration for 5 min. Then, the overall respiratory capacity of mitochondria was measured in the absence of proton gradient. The basal mitochondrial respiration, which is the difference between the respiration rate of normal condition and the rate after adding the complex I inhibitor rotenone (Sigma, R8875) (1 μM), was determined in cells with different treatments.

Electron microscopy

PC12 cells and tissue slides were treated as indicated and fixed with 2.5% glutaraldehyde and 1% Os_2O_4 for 2 h at 4°C. After dehydration, embedded and staining, the sections were observed under a transmission electron microscope (JEOL, JEM-1011, JAPAN).

Transfection and confocal microscopy assay

The pEGFP-C1-LC3 plasmid was kindly provided by Dr. Quan Chen (Institute of Zoology, Chinese Academy of Sciences). Vector pDsRed-Mito was purchased from Clontech (Cat. No. 632421). Cells were cultured in DMEM medium with 10% fetal bovine serum at 37°C in 5% CO_2. In brief, cells (5×10^4 per well) were seeded in 12-well plate with coverslips and grown to 50% confluence. For each well, a mixture of 50 μL containing 1 μg pEGFP-C1-LC3 vector, 0.1 μg pDsRed-Mito vector and 3 μL FuGENE® HD Transfection Reagent (Roche, 04709705001) was incubated at room temperature for 20 min. Meanwhile, culture medium from cells was removed and washed once with Opti-MEM medium. The DNA-FuGENE® HD Transfection Reagent complex was added to each well, together with an additional 450 μL Opti-MEM. After incubation for 6 h in the CO_2 incubator, 1 mL fresh medium was added to each well. 24 h after transfection, cells were treated with morphine or morphine plus melatonin for 24 h. Intact cells were imaged using the Olympus FluoView™ 1000 confocal microscope (Olympus, Melville, NY, USA) at 488 nm and 563 nm, respectively.

Primary cortical neurons culture and immunocytochemistry

Primary cortical neurons were isolated from mouse E15 embryos and were cultured in Neurobasal® Medium supplement with 2% B27 (Gibco-BRL, 17504). Briefly, neurons were seeded in a 12-well plate with a density of 1×10^5 on a coverslip coated with poly-L-ornithine (Sigma, P4957). After having allowed neurons to attach to the plate for four hours, fresh medium was changed. Neurons were cultured for three days, then were treated with morphine and/or melatonin.

To visualize LC3 puncta and neuronal outgrowth, primary neurons were fixed with 4% paraformaldehyde for 30 min at room temperature, then were permeabilized with 0.4% Triton X-100 for 10 min. After washing with PBS, cells were blocked in 1% BSA for 1 h at 37°C, followed by an incubation with antibody against LC3B (Cell Signaling Technology, #3868; 1:50) or TUBB3 tubulin (Abcam, ab7751; 1:200) at 4°C overnight. Immunoreactivity was detected with FITC-conjugated secondary antibody (1:50; KPL, 172-1506) for 1 h at room temperature. Nuclei were counterstained with 1 μg/mL DAPI (Roche Diagnostics, 10236276001) and the slides were visualized under the confocal microscopy (Olympus).

We also performed immunofluorescence assay for endogenous LC3B in PC12 cells treated with and without morphine, with morphine and melatonin, or with melatonin only. In brief, PC12 cells were cultured to 50% confluence on glass slides in a 12-well plate and were transfected with 0.1 μg pDsRed-mito vector and 3 μL FuGENE® HD Transfection Reagent (Roche, 04709705001) for 24 h. Cells were treated with and without 100 μM morphine, with 100 μM morphine and 100 μM melatonin, or with 100 μM melatonin for 24 h, and were fixed and stained for endogenous LC3B following the above procedure for primary neurons.

Western blot

Cell lysates were prepared using protein lysis buffer (Beyotime, P0013). Protein concentration was determined using a protein assay reagent (Bio-Rad, 500-0006). A total of 30 μg protein was separated by 15% SDS PAGE, and was transferred to a polyvinylidene difluoride membrane (Roche Diagnostics, IPVH00010). The membrane was soaked with 10% (w/v) skim milk for 2 h at room temperature. The membrane was incubated with primary antibodies against LC3B (CST, 1:1000, overnight at 4°C), SQSTM1/p62 (Merck Milipore, 1:1000, overnight at 4°C) and ACTB (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD , TA-09 , 1:10000, overnight at 4°C), followed by the peroxidase-conjugated anti-mouse (KPL, #474-1806, 1:5000) or anti-rabbit (KPL, #474-1506, 1:5000) IgG for 1 h at room temperature. The epitope was visualized using an ECL western blot detection kit (Millipore, WBKLS0500).

Behavioral tests on chronic morphine-treated mice with and without melatonin

Male Kunming mice weighing 25 to 30 g at the beginning of the experiments were obtained from animal facility of Kunming Medical College. Mice were housed in clear plastic cages with free access to water and food in an established animal house of 22±2°C and 50% humidity, with a 12 h light/dark cycle. All mice were acclimatized for at least one week before experiments. Experiments were performed during 9:00 am to 17:00 pm each day. Melatonin was dissolved in 5% ethanol saline (v/v). Mice were randomly divided into four groups: 1) control group (vehicle-saline); 2) morphine group (vehicle-morphine); 3) melatonin-morphine group (melatonin-morphine); and 4) melatonin group (melatonin-saline).

Morphine induced behavioral sensitization was measured in an open-field test using the ENV-510 test environment and Activity Monitor software (Med Associates) on day 0 (baseline) and the following 6 days. Mice were subcutaneously injected morphine (10 mg/kg) immediately before confinement to the test room. The controls were received 0.9% saline (10 mL/kg, s. c (subcutaneously)) in all experiments. Each mouse received injection of morphine or saline 30 min after being injected intraperitoneally (i.p) with melatonin (0.5 or 5 mg/kg) or vehicle (10 mL/kg). For all experiments *in vivo*, data acquisition and analysis were performed with a double-blind, controlled design. The animal care and experimental protocol was approved by the Kunming Institute of Zoology, Chinese Academy of Sciences.

Analgesia was assessed using the radiant heat tail-flick latency (LE 7106 Light beam analgesy-meter, Panlab HARVARD, MA, USA). Mice were placed in Plexiglas cages (9×6×3 cm) on a modified Hargreaves Device. Mice were habituated to the device for 2 min before each test session. A halogen lamp was focused on the tail and withdrawal reflex time was determined by a photocell. We measured tail flick latency at FOCUS 30 (decimal selector of heat intensity) every two days from day 0 (baseline) to day 7. We used 20 sec as a cutoff time to avoid damaging the tail. The analgesic response to morphine was assessed by the tail-flick test 1 h after injection of morphine (10 mg/kg). For melatonin treatment, each mouse was injected intraperitoneally (i.p) with melatonin (0.5 or 5 mg/kg) or vehicle (10 mL/kg) and received a morphine injection 30 min later.

Quantification of serum melatonin levels in patients and normal controls

The concentrations of serum melatonin in normal subjects and heroin addicts at time of withdrawal, 3 and 6 months after withdrawal were measured by melatonin enzyme-linked immunosorbent assay kit (IBL Company, RE54021) according to the manufacturer's instructions. The procedure followed the basic principle for competitive immunoassays and the limit of detection was 1.6 pg/mL.

Statistical analysis

Differences of mtDNA copy number in tissues from rats with and without morphine treatment was computed by unpaired *t*-tests. The levels of serum melatonin, mtDNA copy number and damage in peripheral blood of healthy individuals and heroin addicts were also compared using unpaired *t*-tests. We used paired *t*-tests to quantify the difference of serum melatonin and mtDNA alterations in patients at the time of drug withdrawal for 0, 3, and 6 months. All statistical analyses were two-tailed and with 95% confidence intervals (CI) using GraphPad prism 4. Differences in animal behavioral sensitization and analgesic tolerance were determined by ANOVA, followed by LSD (Least Significant Difference) for post-hoc comparisons.

Supplementary References

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8. Wang D, Su LY, Zhang AM, Li YY, Li XA, Chen LL, et al. Mitochondrial DNA copy number, but not haplogroup, confers a genetic susceptibility to leprosy in Han Chinese from southwest China. PLoS One 2012; 7:e38848.

Supplementary Tables

Table S1. Primers for measuring mtDNA copy number and damage in human peripheral blood.

Primer	Sequence (5'-3')	Product length (bp)	Reference
Primers for mtDNA copy number [a]			
hmtDNAF	CCCTAACACCAGCCTAACCA	55	5
hmtDNAR	AAAGTGCATACCGCCAAAAG		
hHBBF	CTATGGGACGCTTGATGT	113	7, 8
hHBBR	GCAATCATTCGTCTGTTT		
Primers for mtDNA damage			
hlmtDNAF	CTGTTCTTTCATGGGGAAGC	972	5
hlmtDNAR	AAAGTGCATACCGCCAAAAG		
hsmtDNAF	CCCTAACACCAGCCTAACCA	55	5
hsmtDNAR	AAAGTGCATACCGCCAAAAG		

[a] mtDNA copy number was quantified relative to the nuclear *β-globin* gene (*Hbb*).

Table S2. Primers for measuring mtDNA copy number in rat and mouse.

Primer [a]	Sequence (5'-3')	Product length (bp)
For rat mtDNA		
rsmtDNAF	ACACCAAGGTTAATGTAGC	62
rsmtDNAR	TTGAATCCATCTAAGCATT	
rHBBF	CAGTACTTTAAGTTGGAAACG	125
rHBBR	ATCAACATAATTGCAGAGC	
For mouse mtDNA		
msmtDNAF	GCCCATGACCAACATAACTG	81
msmtDNAR	CCTTGACGGCTATGTTGATG	
mHBBF	AGGCAGAGGCAGGCAGAT	105
mHBBR	GGCGGGAGGTTTGAGACA	

[a] mtDNA copy number was quantified relative to the nuclear *β-globin* gene (*Hbb*).

Table S3. Real-time PCR assay for genes related to mitochondrial transcription and replication, and autophagy.

Primer	Sequence (5'-3')	Gene	Alias	Product length (bp)
Primers for mitochondrial transcription and replicated genes				
rSsbp1F	GTTCAGTTACTTGGGCGAG	*Ssbp1*	*mtSSB*	129
rSsbp1R	CATTTGGTATGCTTCATTGTC			
rPolgF	GCATCAGCAGAGCCTTG	*Polg*	-	208
rPolgR	TAGCGGTCCTCCTCACG			
rNrf1F	ATGGCGGAAGTAATGAAAGTCA	*Nrf1*	*Nfe2l1_retired*	151
rNrf1R	GTTGCTGTGGCGAGT			
rPolrmtF	CGTGACTTTCGTCTCTGTGC	*Polrmt*	-	172
rPolrmtR	GGGCTTTAGGGACTTGGAG			
Primers for autophagy-related genes				
rAtg3F	GGCTGTTTGGCTATGATG	*Atg 3*	*Apg3l*	131
rAtg3R	ACACATAAGAGGTGGTGGG			
rAtg5F	TGAGTGTGCTCCACCGC	*Atg 5*	-	112
rAtg5R	ATTTCCAATGTTTTCACTGAG			
rAtg7F	AGCCAGCAAGCGAAAGC	*Atg 7*	*Apg7l*	142
rAtg7R	GCCCAGGTCAGCAGGTG			
rAtg12F	AACAATGTCACTTGGAAAGG	*Atg 12*	*Apg12l*	133
rAtg12R	CATCGCAAATGAATACCAG			
Primers for house-keeping gene for normalization				
rβ-actinF	CAGGGTGTGATGGTGGGTATG	*β-actin*	-	124
rβ-actinR	GACAATGCCGTGTTCAATGG			

Supplementary Figures and Figure Legends

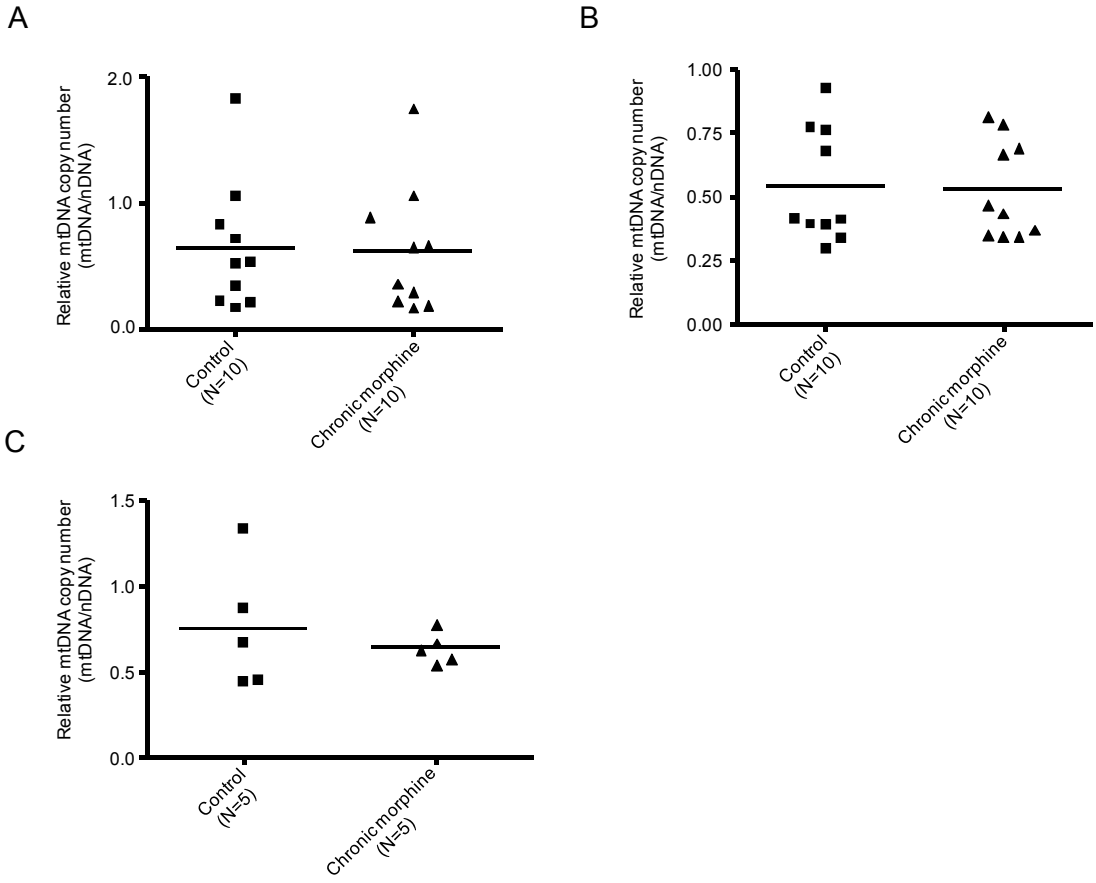

Figure S1. No change of mtDNA copy number in tissues of cortex (A), heart (B), and liver (C) from rats with and without chronic morphine treatment.

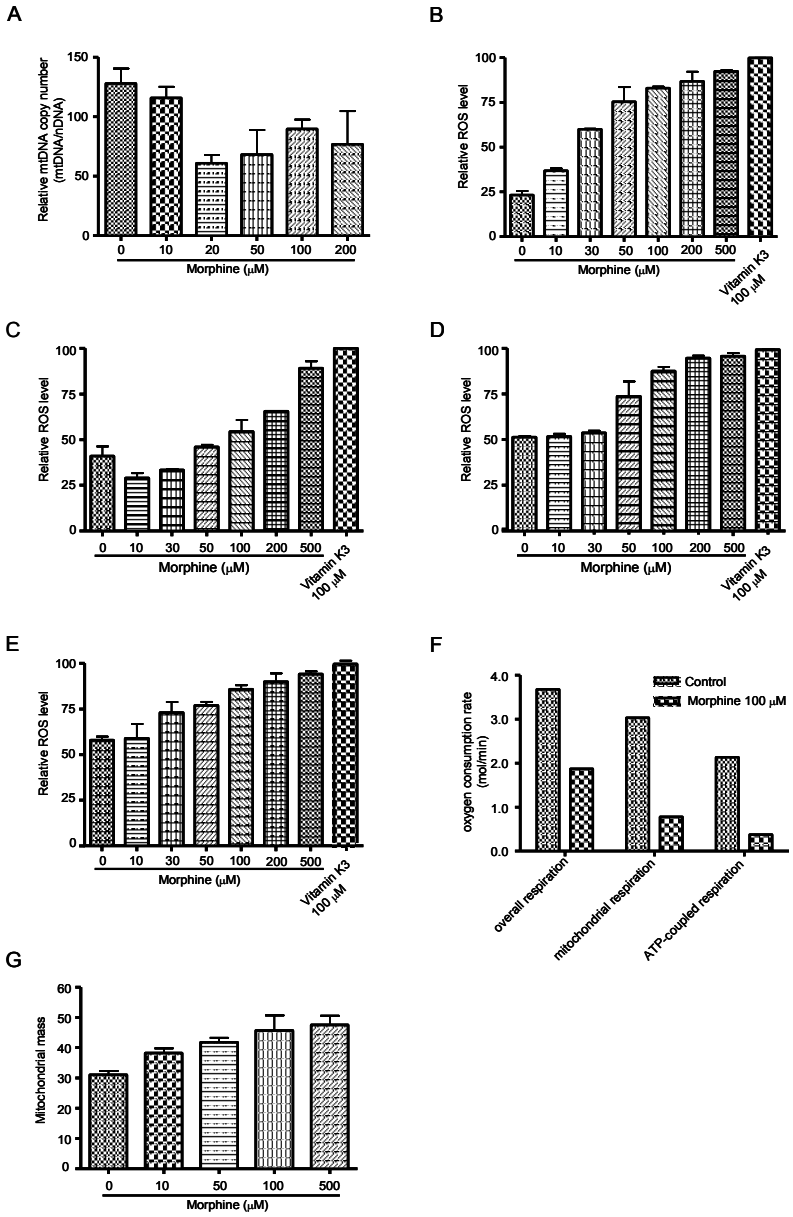

Figure S2. Morphine induces mitochondrial dysfunction and oxidative stress in cultured PC12 cells. PC12 cells were cultured in 5% FBS DMEM medium and were treated with different concentrations of morphine for 24 h. Along with the depletion of mtDNA copy number (A), there was a dose-dependent increase of intracellular ROS level (B). The increase of intracellular ROS induced by morphine was also evidently at 12 h (C), 36 h (D) and 48 h (E), with a dose-dependent manner. Vit-K3 was used as a positive control for generating ROS. Treatment of morphine (100 μM) for 24 h caused a decrease of mitochondrial respiration rate (F). We observed a compensatory increase of mitochondrial mass (G) in cells treated with different concentrations of morphine for 24 h. The experiments were repeated for at least four times and we obtained consistent results.

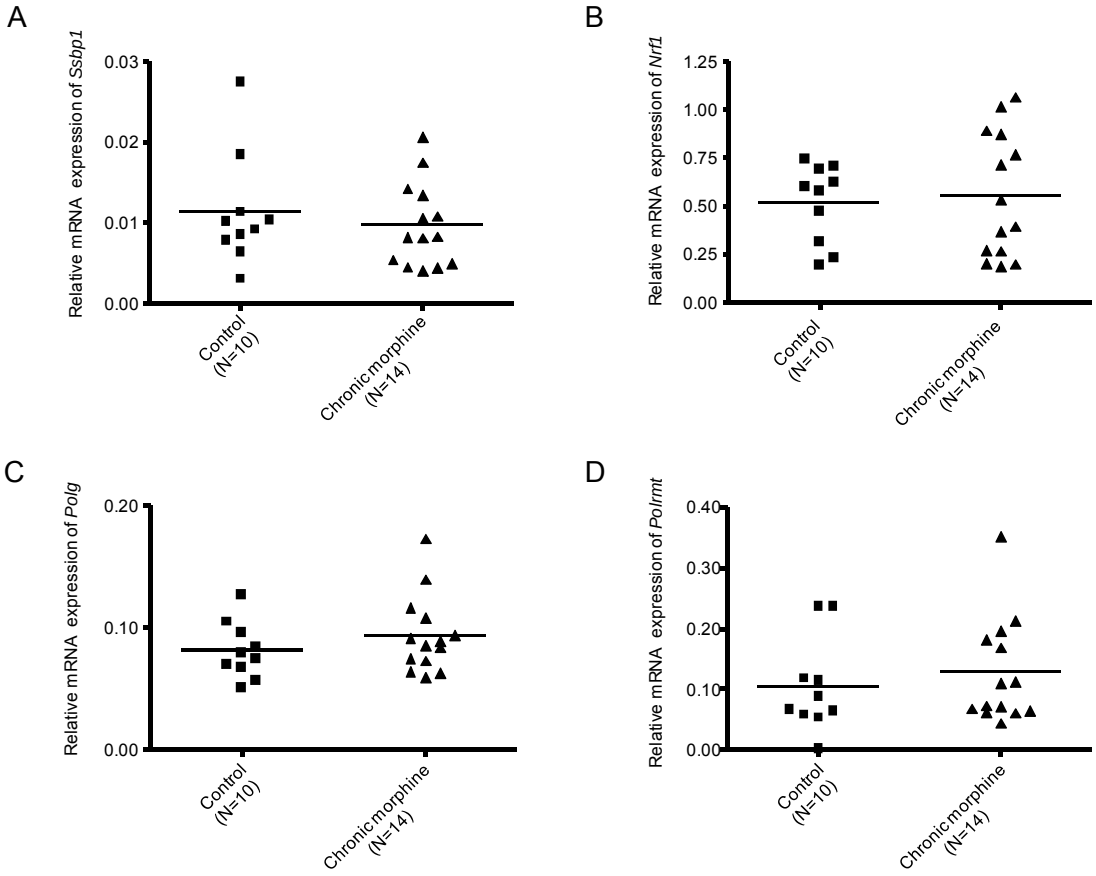

Figure S3. Measurement of mRNA expression levels of genes controlling mitochondrial transcription and replication in hippocampus tissues from rats with and without morphine treatment. There is no difference of the relative mRNA expression level of the *Ssbp1* (A), *Nrf1* (B), *Polg* (C) and *Polrmt* (D) genes between the control (n=10) and morphine-treated (n=14) rats.

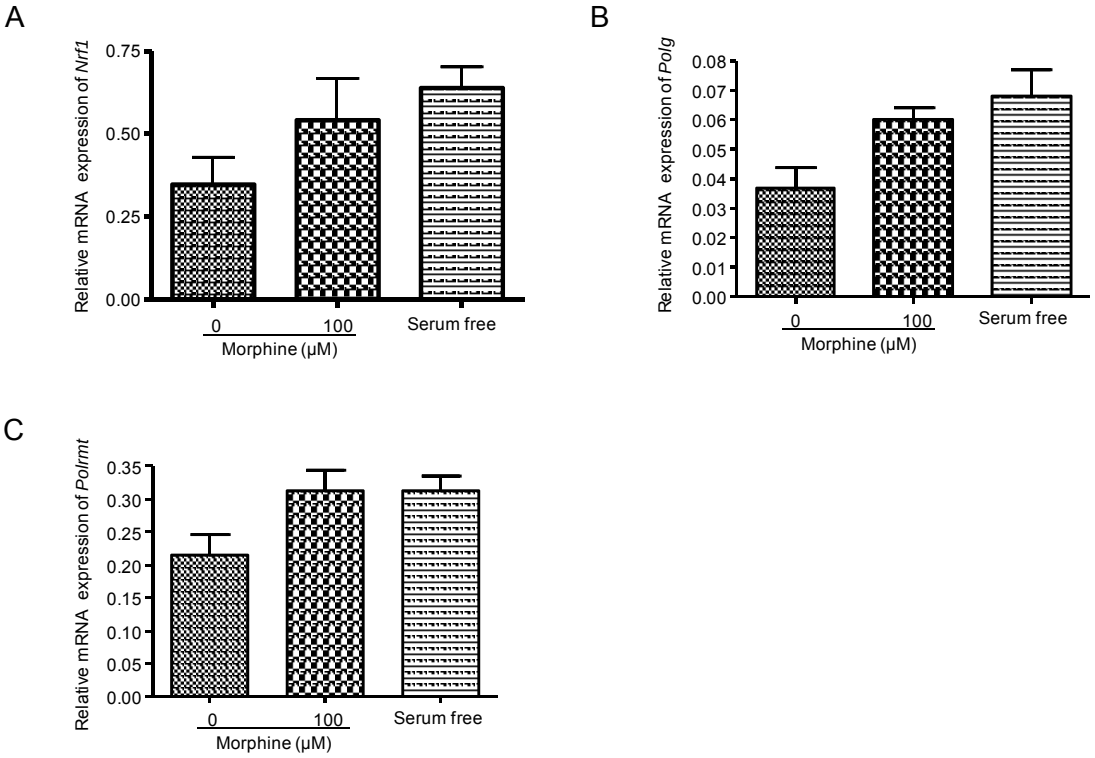

Figure S4. Measurement of mRNA expression levels of *Nrf1* (A), *Polg* (B) and *Polrmt* (C) genes in C6 cells treated with and without morphine and serum starvation. C6 cells received morphine (100 μM) treatment or serum starvation for 12 h had an increase of relative mRNA expression of *Nrf1* (A), *Polg* (B) and *Polrmt* (C) genes. Each treatment contains three wells of the 12-well plate. Data are expressed as means ± STD of a representative experiment. We obtained consistent results based on two independent experiments.

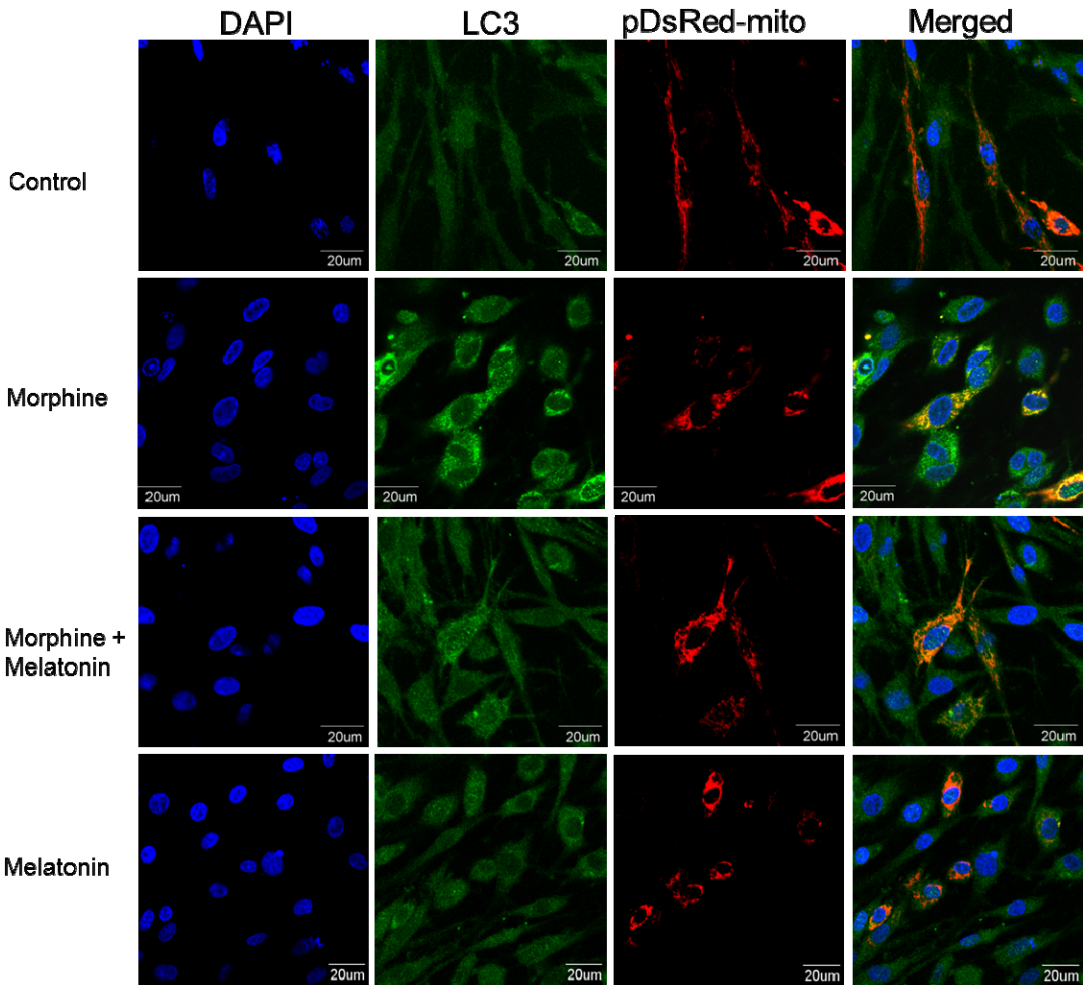

Figure S5. Measurement of endogenous LC3 puncta in PC12 cells by immunofluorescence assay. PC12 cells were cultured in DMEM medium supplemented with 5% FBS to reach 50% confluence on glass slides in a 12-well plate and were transfected with 0.1 μg pDsRed-mito vector and 3 μL FuGENE® HD Transfection Reagent (Roche, 04709705001) for 24 h. Cells were treated with and without 100 μM morphine, with 100 μM morphine and 100 μM melatonin, or with 100 μM melatonin for 24 h, and were fixed and stained for endogenous LC3B. The endogenous LC3 puncta were induced by morphine, which could be rescued by melatonin.

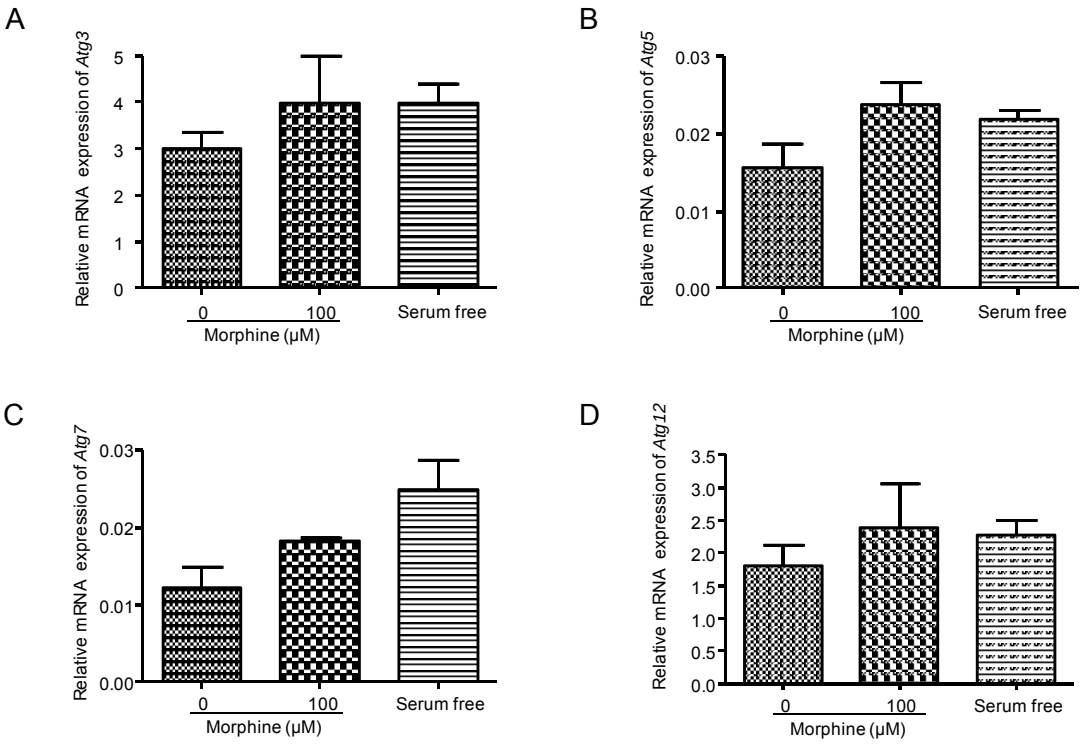

Figure S6. Measurement of mRNA expression levels of autophagy-related genes in C6 cells. C6 cells were cultured in 5%FBS DMEM medium and received treatment with morphine or serum starvation for 12 h. The relative mRNA expression of *Atg3* (A), *Atg5* (B), *Atg7* (C), and *Atg12* (D) genes were increased in C6 cells with morphine (100 μM) treatment and serum starvation compared to the non-treated cells. Each treatment contains three wells of the 12-well plate. Data are expressed as means ± STD of a representative experiment. We obtained consistent results based on two independent experiments.

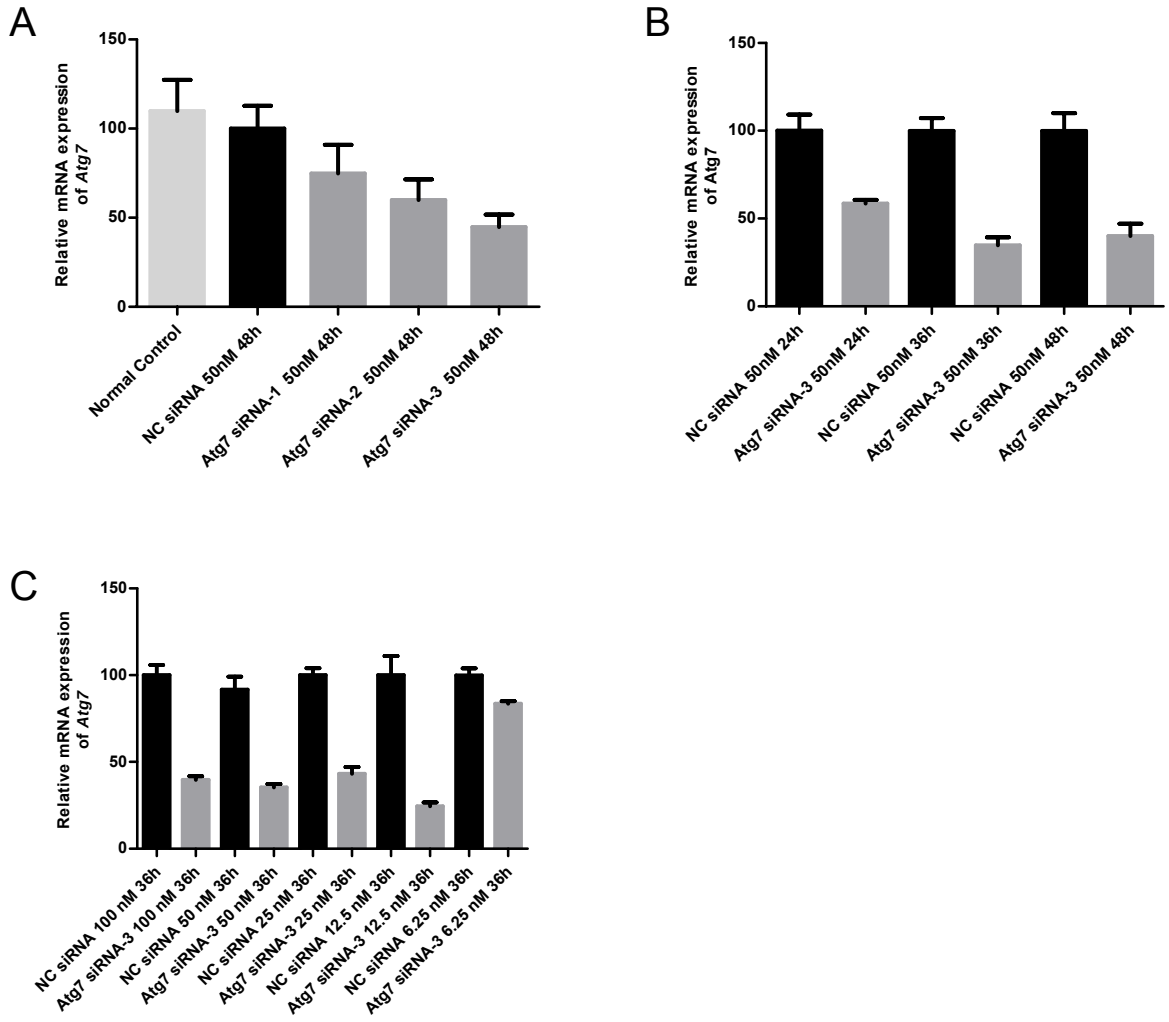

Figure S7. The siRNA knockdown effect of the *Atg7* gene in rat C6 cells. C6 cells were cultured in 5%FBS DMEM medium and transfected with three siRNA oligonucleotides against the *Atg7* gene. The *Atg7* siRNA-3 was the most efficient and was selected for the following experiments (A). The optimum knockdown time of the *Atg7* siRNA-3 was 36 h (B) and the most effective concentration was 12.5 nM (C). Each treatment contains three wells in a 12-well plate. Data are expressed as mean ± SEM of a representative experiment. We obtained consistent results based on two independent experiments.

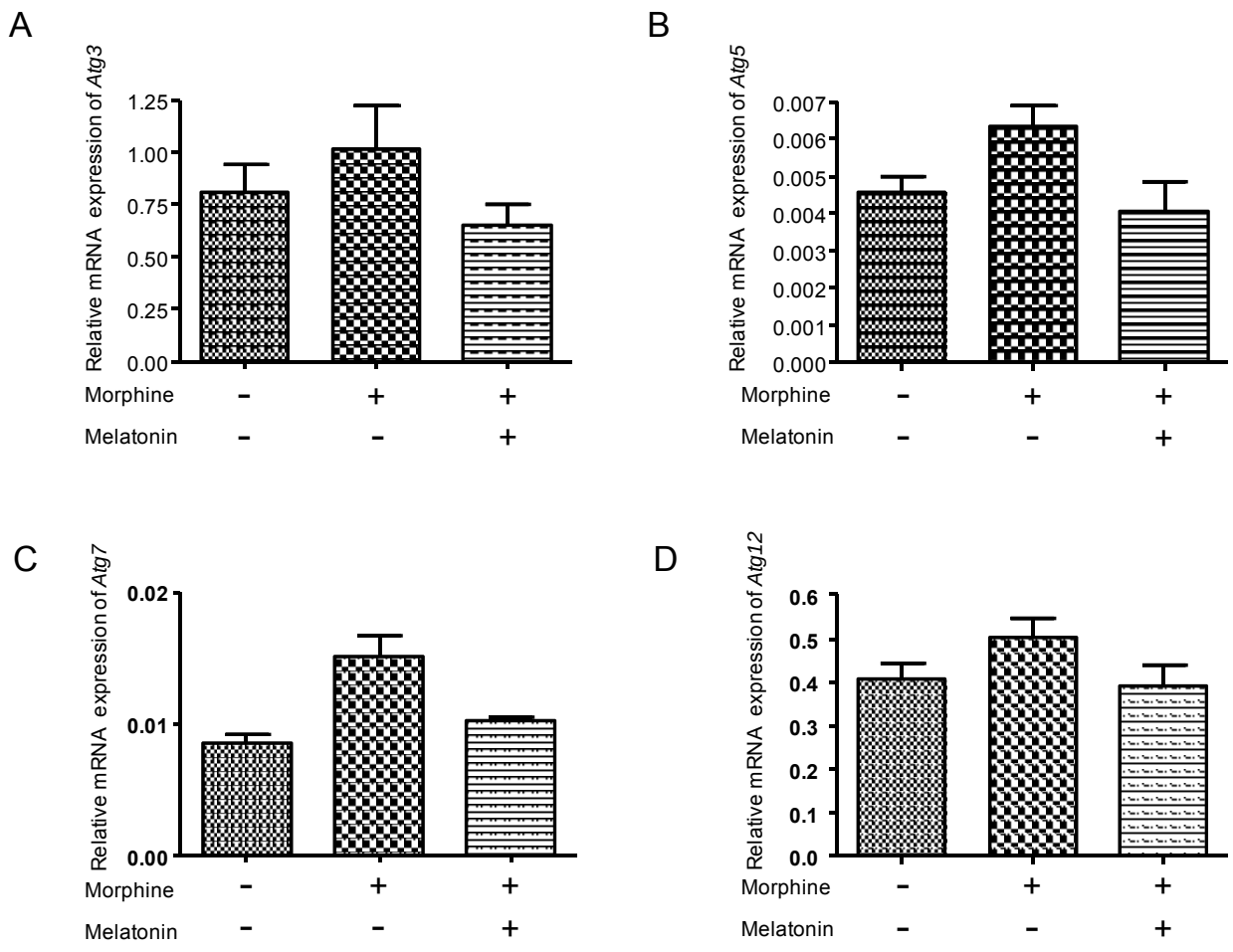

Figure S8. Melatonin restores the increased mRNA expression of *Atg3* (A), *Atg5* (B). *Atg7* (C), and *Atg12* (D) genes in C6 cells treated with morphine. C6 cells were cultured in DMEM supplemented with 5% FBS. Cells with 80% confluence received morphine (100 μM) treatment for 12 h before the harvest. Each treatment contains three wells of the 12-well plate. Data are expressed as means ± STD of a representative experiment. The experiments were repeated twice and we obtained consistent results.

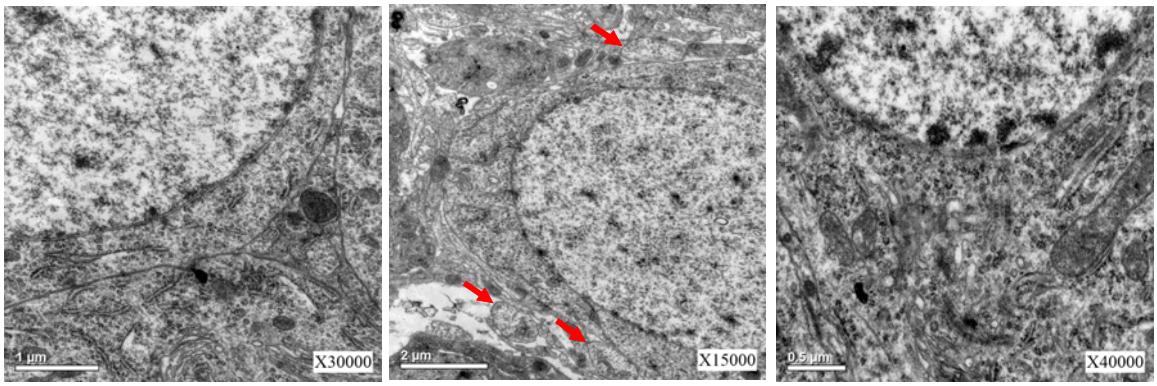

Figure S9. Electron micrographs of hippocampus tissues from mice after behavioral sensitization and analgesic tolerance tests. Arrows point to distinct autophagic structures showing more vacuolar components and secondary-lysosomes.

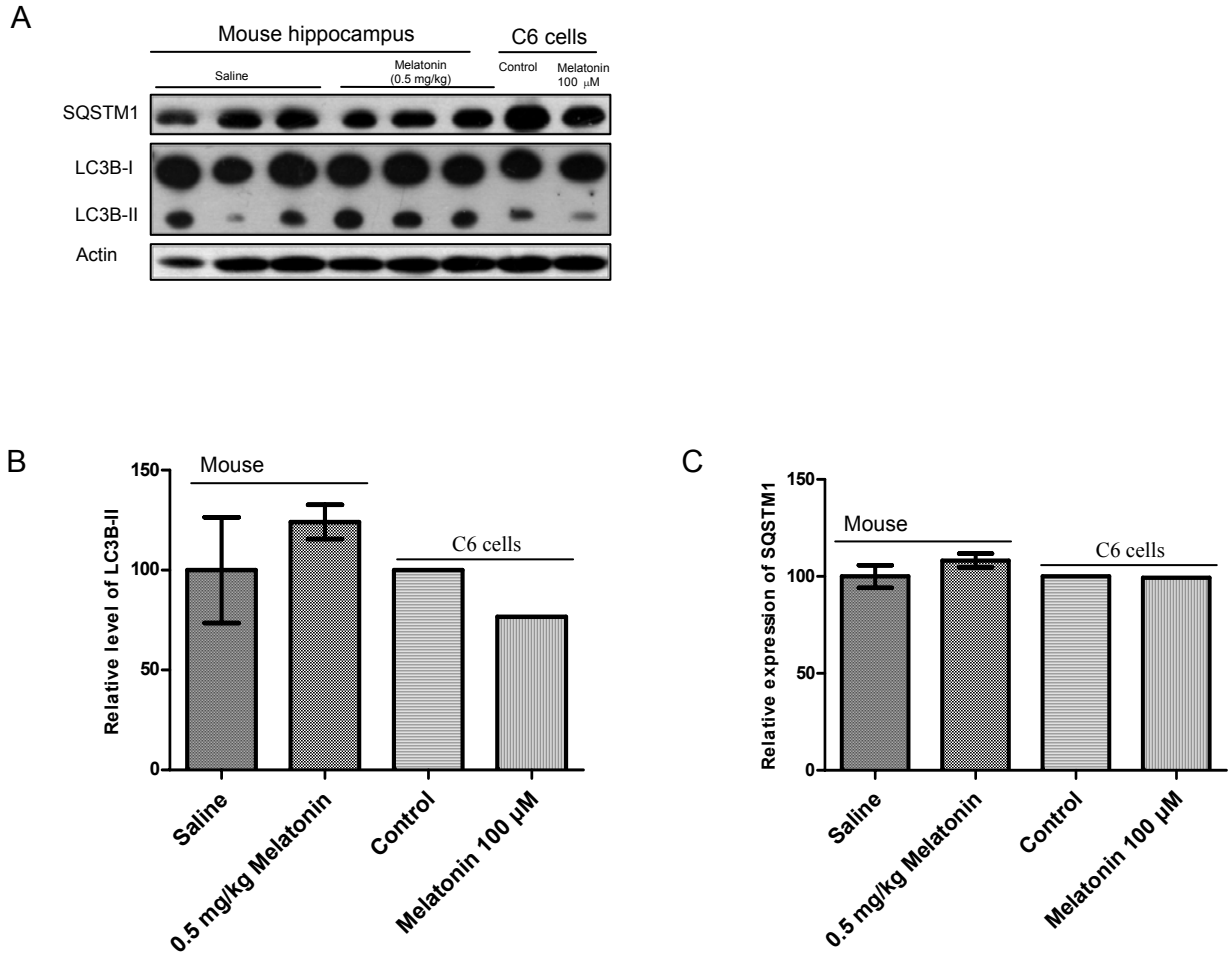

Figure S10. Effect of melatonin on autophagic activation. Melatonin has a seemingly conflicting effect on regulating the level of LC3B-II in mice hippocampus tissues and in C6 cells, but it has no obvious effect on the expression of SQSTM1 (A). Quantification of the levels of LC3B-II (B) and SQSTM1 (C) in hippocampus tissues from mouse receiving an intraperitoneal injection of melatonin (0.5 mg/kg) and C6 cells with and without melatonin treatment (100 μM).