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RESEARCH PAPER

Involvement of neuronal TGF-β activated kinase 1 in the development of tolerance to morphine-induced antinociception in rat spinal cord

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BACKGROUND AND PURPOSE

Tolerance induced by morphine and other opiates remains a major unresolved problem in the clinical management of pain. There is now good evidence for the importance of MAPKs in morphine-induced antinociceptive tolerance. A member of the MAPK kinase kinase family, TGF-β activated kinase 1 (TAK1) is the common upstream kinase of MAPKs. Here, we have assessed the involvement of TAK1 in the development of tolerance to morphine-induced analgesia.

EXPERIMENTAL APPROACH

The effects of an antagonist of TAK1 on morphine tolerance were investigated *in vivo* using the Randall–Selitto test, and the mechanism was investigated using Western blot and immunohistochemistry. The expression of TAK1 after chronic morphine exposure was also evaluated *in vitro* by immunohistochemistry.

KEY RESULTS

Chronic intrathecal morphine exposure up-regulated protein levels and phosphorylation of spinal TAK1. TAK1 immunoreactivity was co-localized with the neuronal marker NeuN. Intrathecal administration of 5Z-7-oxozeaenol (OZ), a selective TAK1 inhibitor, attenuated the loss of morphine analgesic potency and morphine-induced TAK1 up-regulation. Furthermore, OZ decreased the up-regulated expression of spinal p38 and JNK after repeated morphine exposure. *In vitro* studies demonstrated that sustained morphine treatment induced TAK1 up-regulation, which was reversed by co-administration of OZ. A bolus injection of OZ showed some reversal of established morphine antinociceptive tolerance.

CONCLUSIONS AND IMPLICATIONS

TAK1 played a pivotal role in the development of morphine-induced antinociceptive tolerance. Modulation of TAK1 activation by the selective inhibitor OZ in the lumbar spinal cord may prove to be an attractive adjuvant therapy to attenuate such tolerance.

Abbreviations

IKKs, IκB kinases; MAPKKK, MAPK kinase kinase; MPE%, percentage maximum possible effect; OZ, 5Z-7-oxozeaenol; TAK1, TGF-β activated kinase 1

Tables of Links

TARGETS
Enzymes
ERK
JNK
p38
TAK1

LIGANDS
Morphine
TGFβ

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Chronic use of opiates not only causes tolerance, but also suppresses immune functions and affects cell proliferation and cell survival (Rogers and Peterson, 2003; Tegeder and Geisslinger, 2004). Chronic morphine administration also leads to morphine-induced hyperalgesia (Lee *et al.*, 2011). Despite considerable progress, the molecular and cellular mechanisms involved in morphine tolerance are complex: receptor desensitization and endocytosis, intracellular signalling hyperactivity, secondary activation of excitatory amino acid receptors, and subsequent intracellular cascades as well as glial activation and the release of pro-inflammatory mediators have been reported to be involved (Mayer *et al.*, 1999; King *et al.*, 2005; Watkins *et al.*, 2005).

The sequential activation of kinases is a common mechanism of signal transduction in many cellular processes. The MAPK signalling cascades include three major groups (ERK, p38 MAPK and JNK) that transduce a broad range of extracellular stimuli into diverse intracellular responses (Seger and Krebs, 1995). ERK is involved in growth factor-mediated cell proliferation. JNK and p38 MAPK respond to various types of stress, including reactive oxygen species, osmotic pressure, TNF-α and endoplasmic reticulum stress, as well as regulating apoptosis, inflammation, and morphogenesis *via* the phosphorylation of various target molecules (Takeda *et al.*, 2011) that belong to the stress-activated protein kinases. Compelling evidence has accumulated showing that MAPKs play important roles in morphine-induced tolerance and dependence (Narita *et al.*, 2002; Fan *et al.*, 2003; Cui *et al.*, 2006; 2008; Liu *et al.*, 2006; Macey *et al.*, 2009; Horvath *et al.*, 2010).

However, p38 MAPK and JNK are thought to share a common upstream kinase, known as TAK1 (Sakurai, 2012). As TAK1 was originally identified as a key regulator of the TGF-β-induced activation of MAPK (Yamaguchi *et al.*, 1995), TAK1 belongs to the MAPK kinase kinase (MAPKKK) family and is an important component of the signalling pathways that culminate in the activation of the transcription factors, NF-κB and activator protein-1 (AP-1), in response to pro-inflammatory cytokines and microbial products (Wald *et al.*, 2001; Mizukami *et al.*, 2002; Shim *et al.*, 2005; Huangfu *et al.*, 2006; Adhikari *et al.*, 2007). Once activated, TAK1 phosphorylates and activates MKK3/6, MKK4/7, and IκB kinases (IKKs), which in turn activate JNK, p38MAPKs and NF-κB, when JNK and p38 MAPK eventually lead to activation of the transcription factor AP-1 (Moriguchi *et al.*, 1996a; Shirakabe *et al.*, 1997; Hanafusa *et al.*, 1999; Ninomiya-Tsuji *et al.*, 1999; Takaesu *et al.*, 2000; Wang *et al.*, 2001; Silverman *et al.*, 2003).

A recent study revealed that neuropathic pain induced by peripheral nerve injury results in TAK1 activation in spinal glial cells, and TAK1 inhibition diminishes nerve injury-induced mechanical hypersensitivity (Katsura *et al.*, 2008). However, common mechanisms between opioid tolerance and neuropathic pain have been proposed (Mao *et al.*, 1995a,b; Mayer *et al.*, 1999). The present study was designed to investigate the hypothesis that TAK1 activation is induced in association with the development of antinociceptive tolerance to morphine, and that inhibition of TAK1 would attenuate morphine-induced tolerance. We also explored the signalling pathways involved in these effects.

Materials and methods

Animals

All animal care and experimental protocols were approved by the Animal Use and Care Committee of the School of Medicine, Shanghai Jiaotong University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 152 animals were used in the experiments described here.

Male Sprague Dawley rats weighing 200–250 g were purchased from the Medical Animal Center of Fudan University (Shanghai, China). Rats were housed in a temperature- and humidity-controlled environment on a 12 h light/dark cycle (lights on at 07:00 h) for 3–7 days to allow acclimatization before experiments and housed individually after catheterization. Food and water were provided *ad libitum*. Efforts were made to minimize suffering and to reduce the number of animals used.

Intrathecal catheterization and drug delivery

Intrathecal catheters implanted as described previously (Snider *et al.*, 1991). After anaesthesia with pentobarbital (50 mg·kg^{-1}, i.p.), an intrathecal PE-10 catheter (Becton Dickinson, San Jose, CA, USA) was inserted into the lumbar sub-

arachnoid as described previously (Xu *et al.*, 2007). To confirm the catheterization, 10 μL of 2% lidocaine was injected through the catheter on the next day, and after injection, rats showing immediate hindlimb paralysis were considered to have successful placement while those showing no paralysis were excluded from the study. Then, they were allowed to recover for 7 days and were habituated to the test environment before behavioural testing.

Drugs or vehicle were injected through the catheter with a micro-syringe (Hamilton, Reno, NV, USA), followed by a flush of saline (10 μL, Baxter Healthcare, New York, NY, USA). Antinociceptive tolerance to morphine was induced by intrathecal administration of morphine (15 μg·day^{-1}) for 7 consecutive days (Zhao *et al.*, 2012). The effect of the TAK1 inhibitor 5Z-7-oxozeaenol (OZ) on antinociceptive tolerance was assessed in rats with chronic morphine injections and concurrent daily treatment with OZ. Thirty minutes before each morphine administration, OZ (0.5, 1, 2, or 3 μg) or vehicle was given intrathecally. The doses of OZ were chosen based on the dose–response curve in preliminary experiments. To evaluate the expression of antinociceptive tolerance, the response to mechanical stimulation was assessed 30 min after an acute dose of intrathecal morphine (15 μg), and the antinociceptive effects of morphine were also assessed in each group. The morphine sulfate (15 μg/10 μL in saline) was from Shenyang First Pharmaceutical Factory (Shenyang, China); OZ (0.5 μg per 7.5 μL in 33.3% DMSO, 1 μg per 7.5 μL in 33.3% DMSO, 2 μg per 7.5 μL in 33.3% DMSO and 3 μg per 7.5 μL in 40% DMSO) was from Tocris Cookson, Inc (Ballwin, MO, USA). DMSO was from Sigma–Aldrich (St. Louis, MO, USA).

A bolus of 3 μg OZ was injected intrathecally on day 0 (24 h before the first morphine injection) or day 6 to test its effects on the prevention and reversal of the development of tolerance to morphine-induced antinociception.

Further details of the treatment of animals are described in the Supporting Information.

Behavioural nociceptive tests

A Randall–Selitto apparatus (37215, Ugo Basile, Italy) was used to measure the paw pressure threshold. Briefly, a cone-shaped probe with a rounded tip (9 mm diameter at the base) was applied to a paw, and then the intensity of stimulation was increased from low to high in steps of 10 g with a cut-off of 250 g to avoid tissue injury. The pressure causing an escape response was defined as the withdrawal threshold and recorded. Training sessions were carried out for 4 consecutive days to increase the sensitivity of the test (Taiwo *et al.*, 1989). All assessments were made twice daily, 30 min after drug injection. The basal latency was measured prior to all treatments.

Preparation of spinal cord total lysate and Western blot analysis

To investigate the time-course of spinal TAK1 protein expression during repeated intrathecal morphine administration, rats were killed on days 1, 3, 5 and 7, and the lumbar cord was collected for Western blot analysis.

After drug treatment, rats were killed by rapid intra-cardiac infusion of ice-cold saline containing heparin under i.p. pentobarbital (50 mg·kg^{-1}) anaesthesia, and the lumbar enlargement was immediately removed and prepared for Western blotting. The dorsal portion of the lumbar enlargement (100 mg) was homogenized in 400 μL ice-cold RIPA buffer (Thermo Scientific, Waltham, MA, USA), and the lysate centrifuged at 12 000× *g* for 10 min at 4°C to isolate proteins. The protein concentrations of the samples were determined with a BCA protein assay kit (Beyotime, Shanghai, China). Samples containing 50 μg protein were denatured by heating at 100°C for 5 min, separated on 10% SDS–polyacrylamide gels, and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween (TBST) for 2 h at room temperature and incubated overnight at 4°C with polyclonal rabbit antibodies against rat TAK1 (#4505s, 1:2000, Cell Signaling Technology, Danvers, MA, USA), p-TAK1 (#ab192443, phospho-T187, 1:1000, Abcam, Cambridge, MA, USA), p38 MAPK (#9212, 1:1000, Cell Signaling Technology), JNK (#9258, 1:1000, Cell Signaling Technology), phospho-P38 MAPK (#4511, 1:1000, Cell Signaling Technology), phospho-JNK (#4668, 1:1000, Cell Signaling Technology), or mouse anti-β-actin (1:2000, Beyotime). All primary antibodies were diluted in 5% non-fat milk in TBST buffered saline. Then, the membranes were washed with TBST and incubated for 2 h at room temperature with HRP-conjugated secondary antibodies, as appropriate (1:2,000 in 5% non-fat milk in TBST buffered saline) (Pierce Chemical Co., Rockford, IL, USA). Signals were finally detected using enhanced chemiluminescence reagent (ECL, Thermo Fisher Scientific, Rockford, IL, USA), and visualized with the ChemiDocXRS system (Bio-Rad, Hercules, CA, USA). All Western blot analyses were performed at least three times, and consistent results were obtained.

Fluorescence immunohistochemistry and image analysis

For fluorescence immunohistochemistry, rats were anaesthetized and transcardially perfused with 4% cold paraformaldehyde on day 7. Lumbar spinal cords were harvested, post-fixed for 4 h at 4°C in 4% paraformaldehyde, and then cryoprotected sequentially in 10, 20 and 30% sucrose overnight for 3 days. Frozen sections (35 μm) were cut on a cryostat and air-dried on microscope slides for 30 min at room temperature. For dual antibody immunofluorescence, primary antibodies against TAK1 (#sc-7162, rabbit anti-rat, 1:50 dilution in PBS; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Iba-1 (#ab5076, goat anti-rat, 1:200 dilution in PBS, Abcam), GFAP (#3655, mouse anti-rat, 1:2000 dilution in PBS, Cell Signaling Technology), or NeuN (#MAB377, mouse anti-rat, 1:2000 dilution in PBS, Chemicon, Billerica, MA, USA) were incubated with the tissue sections in 1% normal donkey serum and 0.01% Triton-X-100 (Sigma–Aldrich, St. Louis, MO, USA) overnight at 4°C. The appropriate fluorescent secondary antibody (1:500, Alexa Fluor 488 or 567; Invitrogen, Inc., Carlsbad, CA, USA) was used for each primary antibody. Confocal microscopy of dual antibody immunofluorescence in the dorsal horn was performed with a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan). TAK1-positive cells were counted under a 20× objective.

Primary neuronal cell culture and drug treatments

Pregnant rats were killed on day 14.5 of gestation (the mating day was considered as day 0.5 of gestation) under deep anaes-

thesia. The spinal cords of the embryonic rats were removed aseptically, collected in cold HBSS (Gibco, Grand Island, NY, USA), and then digested in 0.05% trypsin (Invitrogen) diluted in HBSS at 37°C for 15 min. The cell suspension was filtered and centrifuged at 1000 rpm for 4 min at room temperature. Neurobasal medium (Gibco) containing 10% B27 supplement (Gibco), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Invitrogen) were added. Cells were seeded in polylysine-pretreated 24-well plates, yielding a density of 1.8–2.0 × 10^5 cells per well. Groups were divided according to drug treatment: control, morphine, 0.1 μM OZ plus morphine, 0.3 μM OZ plus morphine, 1 μM OZ plus morphine, 0.1 μM OZ, 0.3 μM OZ, 1 μM OZ, and 0.1% DMSO groups. In the control group, a volume of saline the same as that in the morphine group was added into the culture. In the morphine group, morphine was diluted with culture medium, and the final concentration in each well was 10 μM. In the inhibitor plus morphine group, OZ was added so that final concentration in each well was 0.1, 0.3 or 1.0 μM. After incubation at 37°C for 30 min, 10 μM morphine was added. In the vehicle/inhibitor groups, volumes of vehicle/inhibitor the same as the morphine group were added into the culture. The culture medium was changed every other day. All drug treatments lasted for 6 days. Cell immunofluorescence was performed to investigate TAK1 expression as well as co-localization.

Cell immunofluorescence

The cells were fixed in 4% paraformaldehyde at room temperature for 15 min, and then blocked with 10% normal donkey serum in 0.01 M PBS at room temperature for the next 30 min. Next, the cells were incubated with primary antibodies: TAK1 (rabbit anti-rat, 1:50, Santa Cruz Biotechnology) and NeuN (mouse anti-rat, 1:2000, Chemicon) overnight at 4°C. The appropriate fluorescent secondary antibodies (1:500, Alexa Fluor 488 or 567; Invitrogen) were used for fluorescent staining. The antibodies mentioned earlier were diluted in 0.01 M PBS containing 1% normal donkey serum and 0.01% Triton-X-100. Images were captured using a laser confocal microscope (FV1000; Olympus), and laser-scanning settings were controlled to semi-quantify TAK1 expression; identical settings were used for all slides.

Data analysis

All data are presented as mean ± SEM. The analyses were performed using the Statistical Package for the Social Sciences (SPSS) Statistics 21 software (IBM Corp., Chicago, IL, USA). Mechanical paw-withdrawal threshold data were expressed as a percentage of the maximum possible effect (MPE%). The MPE% was calculated as (response latency – baseline latency)/(cut-off latency – baseline latency) × 100; the post-drug paw-withdrawal threshold was the response measured at a specific time after the initiation of infusion or after an intrathecal dose of the probe drug. All mechanonociceptive data for the Randall–Sellitto tests are presented in the Supporting Information. The appropriate paired *t*-test (two-tailed) or ANOVA was used to determine the statistical significance with a criterion of $P \leq 0.05$. The paw-withdrawal pressure thresholds were analyzed using two-way (time and treatment) ANOVA followed by one-way ANOVA (at each time point) with a *post hoc* Dunnett's test. Analysis of immunoreactivity was by one-way ANOVA, followed by multiple comparisons with the Bonferroni *post hoc* test.

Results

Chronic morphine treatment induces TAK1 activation in the spinal dorsal horn

The paw-withdrawal threshold was measured 30 min after daily intrathecal injection of morphine. Although morphine markedly increased the threshold on day 1, the antinociception was reduced within 3 days of treatment, and by day 7, morphine had no effect (Figure 1A, see original mechanonociceptive data in Table S1), demonstrating the development of antinociceptive tolerance.

We then determined the time-course of TAK1 protein expression after chronic morphine application by Western blot. In parallel with the development of tolerance, the expression of TAK1 protein increased prominently after 3 days of morphine treatment (Figure 1B). Besides, intrathecal administration of morphine for 7 days also resulted in significantly increased TAK1 phosphorylation (Figure 1C). Consistent with the results from immunoblotting, immunohistochemistry showed the same tendency for elevated TAK1 expression. The numbers of TAK1-positive cells in control rats were markedly fewer than those in morphine-treated animals (Figure 1D). Chronic morphine exposure induced a significant increase in TAK1-positive cells (Figure 1D), and the increased TAK1 expression was prominently located in laminae I–III of the dorsal horn (Figure 1D).

TAK1 expression increases in dorsal horn neurons after chronic morphine administration

To clarify which cells specifically express TAK1 in morphine-tolerant conditions, we performed double immunofluorescence staining with cell-specific markers in the spinal cord sections. In the dorsal horn, TAK1-positive cells were predominantly found in laminae I–IV, restrictively double-labelled with the mature neuronal marker NeuN (Figure 2D), but not the astrocytic marker GFAP (Figure 2I) or the microglial marker IBa-1 (Figure 2N). Furthermore, we stained the nuclei with DAPI to determine whether the TAK1 was located in the cytoplasm or the nucleus (Figure 2E/T). The higher-magnification images showed that the TAK1 induced by chronic morphine was localized in clusters on the nucleus (Figure 2T). There was considerable background staining with the TAK1 antibody, and this was taken into account when considering co-localization.

Pharmacological blockade of TAK1 prevents morphine antinociceptive tolerance and suppresses TAK1 activation

The effect of OZ, the selective TAK1 inhibitor, on morphine tolerance was assessed using the Randall–Selitto test. We applied intrathecal OZ at different doses (0.5, 1, 2, or 3 μg) 30 min before daily morphine injections. Morphine administration induced significant acute increases in the paw-withdrawal threshold to mechanical stimulation and a total loss of the antinociceptive efficacy occurred by day 7 (Figure 1A), while daily OZ co-administration with morphine maintained the antinociceptive efficacy and prevented the development of antinociceptive tolerance. Compared with

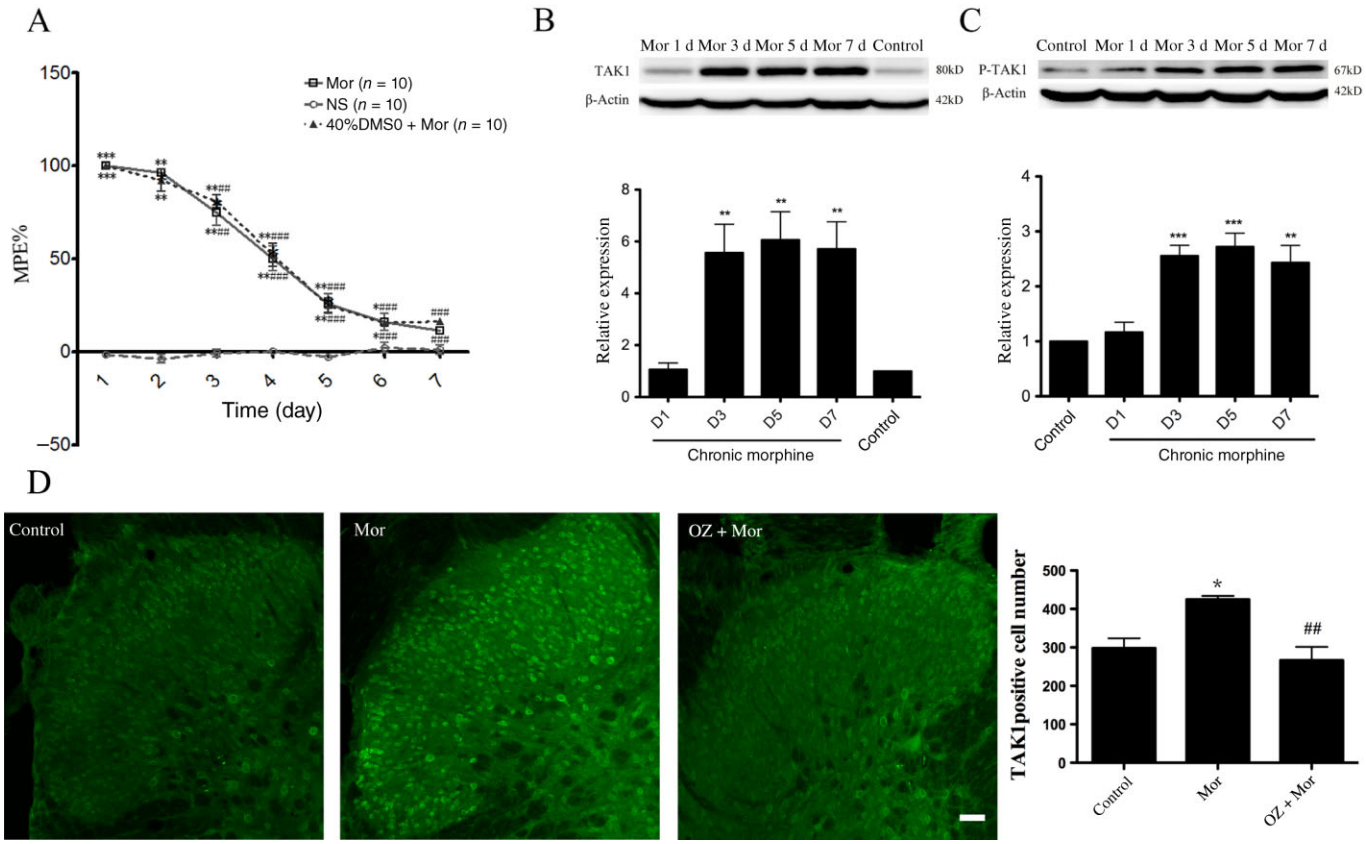

Figure 1

Repeated intrathecal morphine (Mor) induces antinociceptive tolerance to mechanical stimuli and alters TAK1 expression and phosphorylation in homogenates from the spinal dorsal horn. Mechanical paw withdraw threshold data are expressed as MPE%. Values are expressed as mean % ± SEM. (A) The development of morphine antinociceptive tolerance to mechanical stimuli measured with an analgesia meter. NS, normal saline. (B) Relative expression of total spinal TAK1 during repeated morphine exposure ($n = 10$). (C) Relative expression of spinal phosphorylated TAK1 after repeated morphine administration ($n = 10$). (D) Images of TAK1 immunofluorescence in saline, morphine alone, and TAK1 inhibitor OZ plus morphine, and counts of TAK1-positive cells in the superficial dorsal horn on day 7; $n = 5$, 5 images per animal. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significantly different from morphine group; two-way and one-way ANOVA. Scale bar, 50 μm; NS, normal saline.

the morphine group, the 0.5 μg OZ plus morphine group showed an alleviation of the decreased analgesic effect on days 4 and 5, but had no effect as morphine continued to be administered (Figure 3A). Co-administration of 1 μg OZ with morphine for 7 consecutive days, however, greatly attenuated the development of tolerance and retained the antinociceptive effect of morphine throughout the treatment (Figure 3B). Even higher doses (2 and 3 μg) also showed prominent potency in attenuating the development of tolerance (Figure 3C, D). No differences were detected among the 1, 2 and 3 μg OZ plus morphine groups (Figure 3E). Intrathecal delivery of 1 μg OZ alone did not have any significant effect on the basal paw-withdrawal response (Figure 3E) and even 40% DMSO had no affect on the development of morphine tolerance (Figure 1A). Parallel to the behavioural changes, the immunohistochemical data showed that co-administration of 1 μg OZ reduced the TAK1-positive cells in the dorsal horn induced by chronic morphine on day 7 (Figure 1D). These results were further confirmed by Western blot (Figure 4). Original mechanonociceptive data were shown in Supporting Information Table S2.

Based on the behavioural results, we then assessed the protein levels of TAK1 in the OZ plus morphine groups. Interestingly, except for the 0.5 μg OZ plus morphine group, 7 days of intrathecal injection of OZ (1, 2 or 3 μg) suppressed the morphine-induced expression of TAK1 as well as its phosphorylation level (Figure 4A and B). So, the activation of TAK1 induced by chronic morphine was inhibited by intrathecal OZ.

Inhibition of TAK1 by intrathecal OZ reduces the increased expression of MAPKs induced by chronic morphine

We then explored whether the activation of TAK1 influences the increase of MAPKs induced by chronic morphine. Consistent with a previous study showing that chronic morphine activates microglial p38 MAPK in the spinal cord (Cui *et al.*, 2006), the expression of phosphorylated p38 MAPK was up-regulated after chronic morphine exposure in the morphine alone and morphine plus DMSO groups (Figure 5A). But we did not find this in microglia. Interestingly, the increased phosphorylation level of p38 MAPK protein was

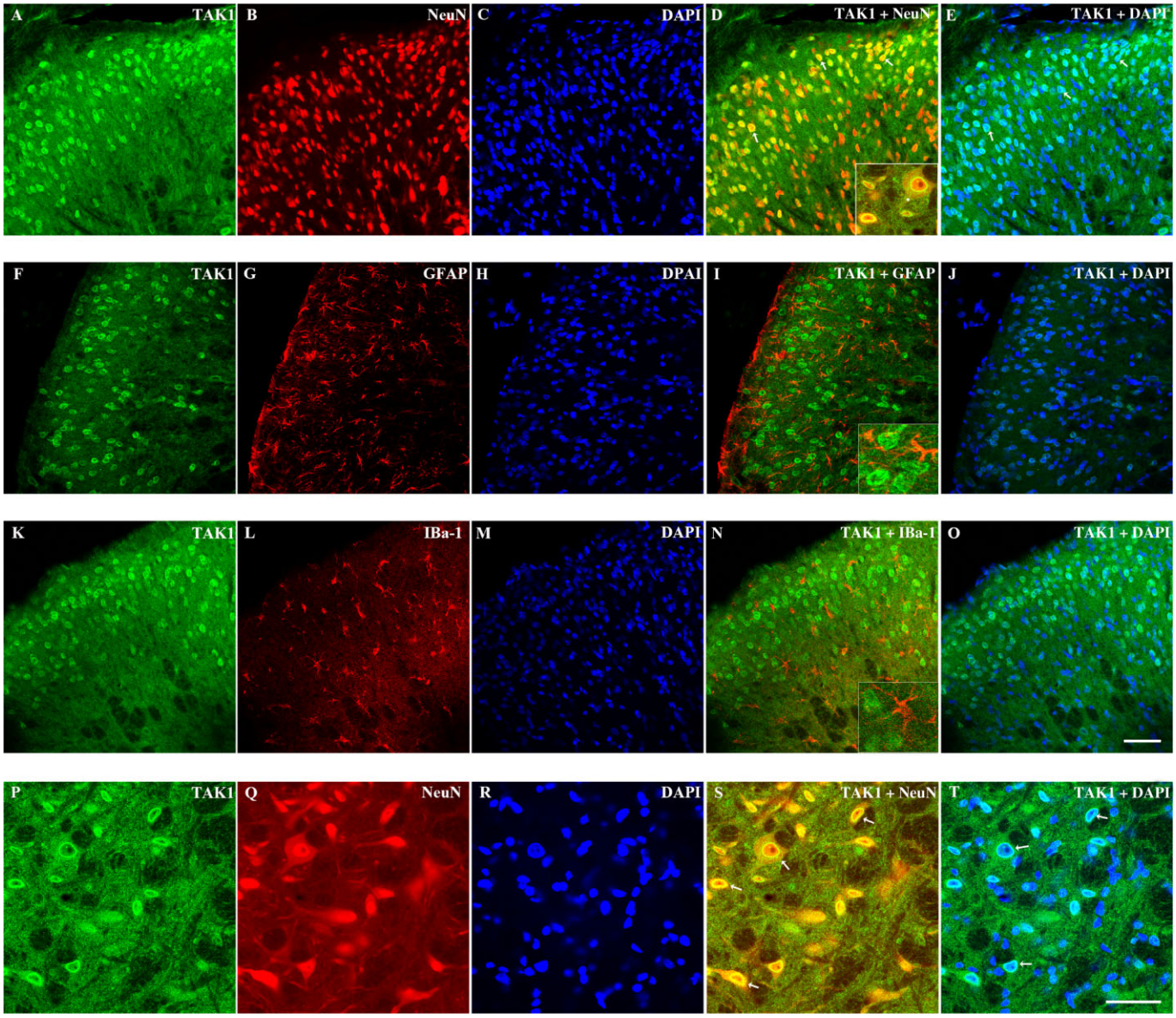

Figure 2

Morphine-induced TAK1 up-regulation is expressed in spinal neurons. (A–E) Confocal images of TAK1 (green) and NeuN (red) immunofluorescence co-localization in neuronal processes (arrows) (40× magnification). (F–J) Immunofluorescence images of TAK1 (green) and GFAP (red) with no co-localization (40× magnification). (K–O) Immunofluorescence images of TAK1 (green) and Iba-1 (red) with no co-localization (40× magnification). (P–T) Confocal images of TAK1 (green) and NeuN (red) co-localization in neuronal cell nuclei (blue) after chronic morphine exposure (arrows) (60× magnification). All spinal cords were harvested on day 7 after chronic morphine exposure. Scale bars, 50 μm.

reversed by co-administration of morphine and 1 μg OZ (Figure 5A), indicating that OZ might prevent the development of tolerance by inhibiting the up-regulation of p38 MAPK phosphorylation.

Similarly, chronic morphine exposure caused a significant increase in JNK phosphorylation in the morphine alone and morphine plus DMSO groups (Figure 5B). The TAK1 inhibitor had an inhibitory effect on the morphine-induced up-regulation of phosphorylated JNK when co-administered with morphine (Figure 5B).

Bolus intrathecal injection of TAK1 inhibitor prevents the development of tolerance to morphine-induced antinociception and reverses established tolerance

To further explore the role of TAK1 in morphine antinociceptive tolerance in the spinal cord, we investigated whether a bolus injection of its inhibitor OZ was able to prevent the development of tolerance or reverse established tolerance. Pretreatment with a single dose of 3 μg OZ 24 h before the first morphine injection clearly maintained the antinociceptive

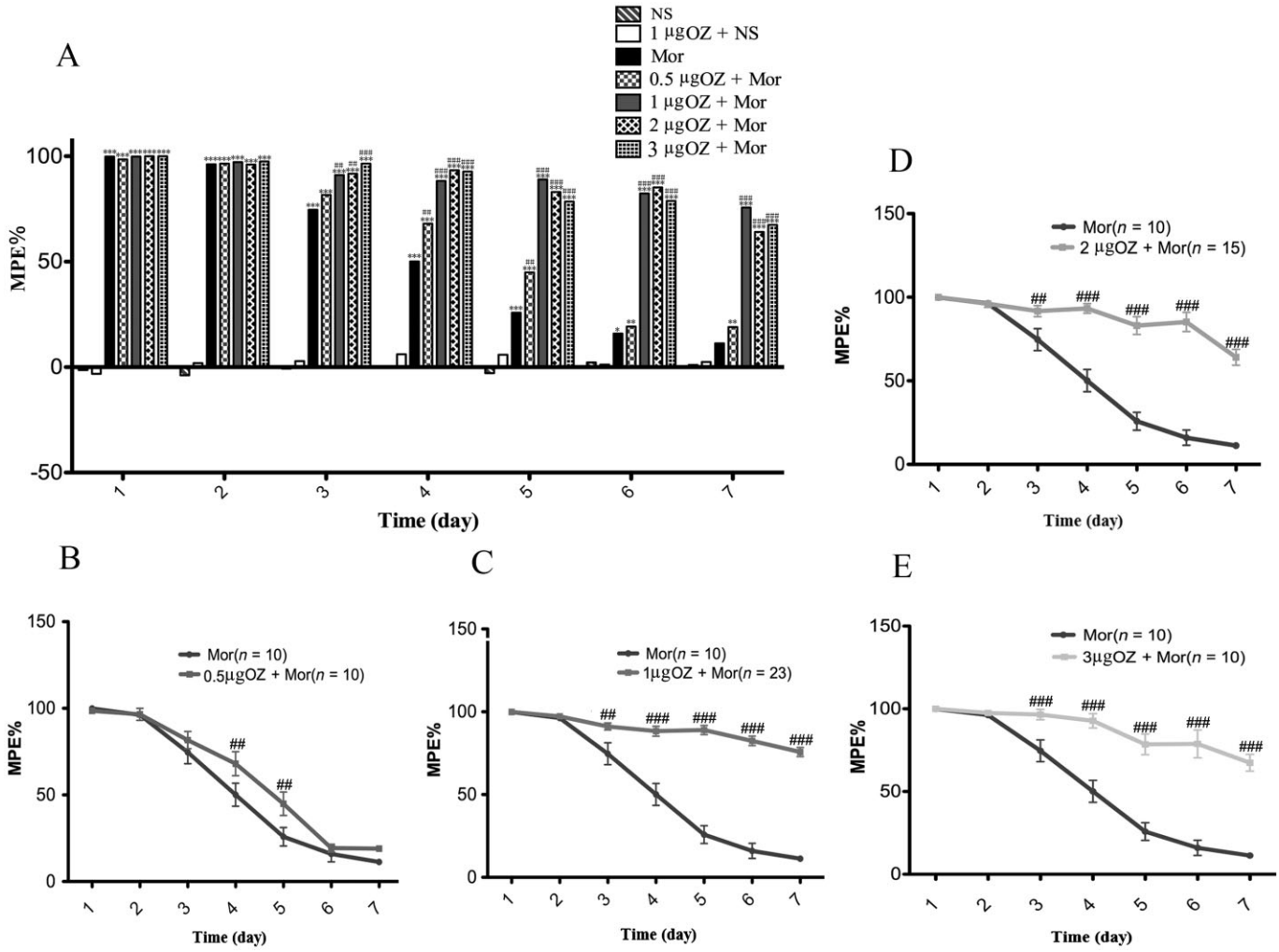

Figure 3

Daily intrathecal injection of a TAK1 inhibitor attenuates morphine-induced antinociceptive tolerance to mechanical stimuli. Mechanical withdrawal latency was measured daily in all treatment groups. Morphine-induced antinociception appeared in all groups receiving chronic intrathecal morphine. Morphine-induced antinociception decreased after daily morphine exposure and antinociceptive tolerance was evident by day 7 (A). The 0.5 μg OZ plus morphine (Mor) group retained the morphine-induced antinociception on days 4 and 5, but this did not prevent the development of tolerance (B). However, 1 μg (C), 2 μg (D) and 3 μg (E) of the TAK1 inhibitor OZ prevented the development of tolerance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significantly different from morphine group; two-way ANOVA.

efficacy of morphine during the 7 consecutive days of administration (Figure 6, see original mechanonociceptive data in Supporting Information Table S3). Furthermore, a single intrathecal dose of 3 μg OZ on day 6 markedly restored the antinociceptive efficacy of morphine. Intrathecal injection of 15 μg morphine induced marked analgesia in the reversed group on day 7 (Figure 6, see original mechanonociceptive data in Supporting Information Table S3).

OZ inhibits morphine-induced TAK1 activation in primary cultured neuronal cells

Primary neuronal cells were cultured to test the effects of morphine and OZ on TAK1 activity *in vitro*. The cells were stained by incubating then with primary antibody against the neuron-specific marker NeuN, indicating that we had cultured the appropriate cells. Consistent with the results *in vivo*, confocal photomicrographs showed a high degree of co-localization of TAK1 with NeuN in the same cells, and activated TAK1 was mainly expressed in the DAPI-stained nuclei (Figure 7A). Chronic morphine treatment (10 μM) increased the mean fluorescence density of TAK1-positive cells. But this tendency was reversed by co-administration of 0.3 and 1 μM OZ, but not 0.1 μM OZ (Figure 7B and C). OZ alone (0.1, 0.3, 1 μM) and 0.1% DMSO did not affect the mean fluorescence density of TAK1-positive cells (Figure 7B and C).

Discussion

To reveal the mechanisms underlying morphine tolerance, studies have mainly focused on neuronal and microglial involvement, during the past decades. The present study, for

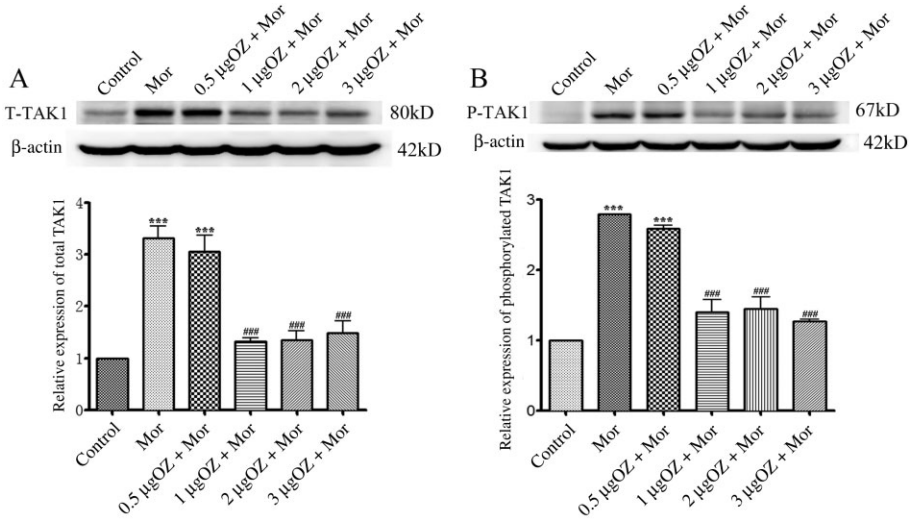

Figure 4

TAK1 inhibitor attenuates morphine-induced TAK1 expression and phosphorylation. Dorsal lumbar spinal cord protein from rats receiving intrathecal saline alone, morphine (Mor) alone and different doses of OZ plus morphine was probed for total and phosphorylated TAK1 and β-actin protein expression. All tissue samples were taken on day 7. Quantification of fold-changes of total (A) or phosphorylated (B) TAK1 expression normalized to β-actin loading control ± SEM is located below representative Western blots; $n = 10$ in each group. $^{***}P < 0.001$, significantly different from control group; $^{###}P < 0.001$, significantly different from morphine group; one-way ANOVA.

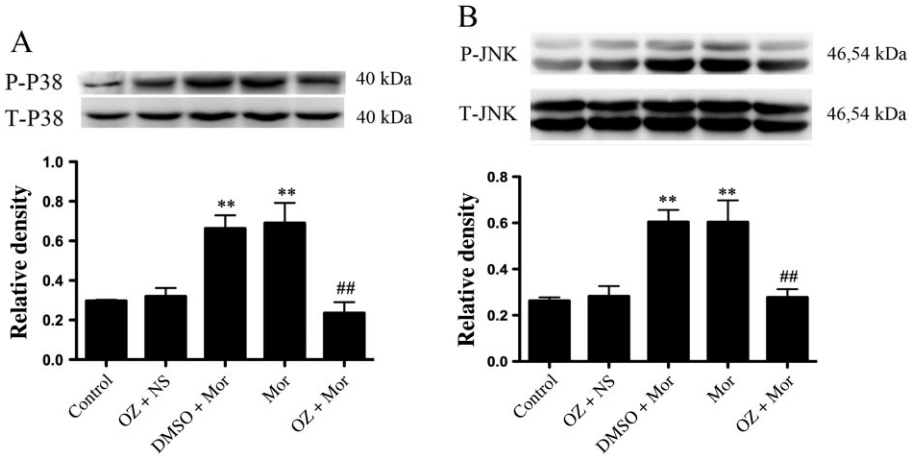

Figure 5

TAK1 inhibitor attenuates morphine-induced up-regulated phosphorylation of spinal p38 MAPK and JNK. Dorsal lumbar spinal cord protein from rats receiving intrathecal saline alone, saline plus OZ (1 μg), DMSO plus morphine (Mor), morphine alone, and OZ (1 μg) plus morphine was probed for total and phosphorylated p38 MAPK and JNK protein expression. Quantification of fold-changes of phosphorylated p38 MAPK (A) and JNK (B) expression normalized to total expression of p38 MAPK and JNK; $n = 10$ in each group. $^{**}P < 0.01$, significantly different from control group; $^{##}P < 0.01$, significantly different from morphine group; one-way ANOVA.

the first time, demonstrates the critical role of TAK1 in the development of morphine antinociceptive tolerance. Here, we investigated the involvement of TAK1 and obtained four major findings: (i) chronic morphine exposure stimulated TAK1 activation *in vivo* and *in vitro*, and intrathecal morphine administration significantly increased TAK1 protein expression and phosphorylation in the spinal dorsal horn; (ii) treatment with the TAK1-selective inhibitor OZ restored the antinociceptive effect of morphine and prevented the development of tolerance to this effect. In line with the behavioural results, OZ suppressed the up-regulated TAK1 protein level as well as phosphorylation when administered intrathecally together with morphine; (iii) inhibition of TAK1, on the other hand, reduced the increase of phosphorylated p38 MAPK and phosphorylated JNK induced by chronic morphine exposure, suggesting that TAK1 influences the morphine tolerance process *via* the p38 MAPK and/or JNK pathways; and (iv) a single dose of 3 μg OZ prevented or

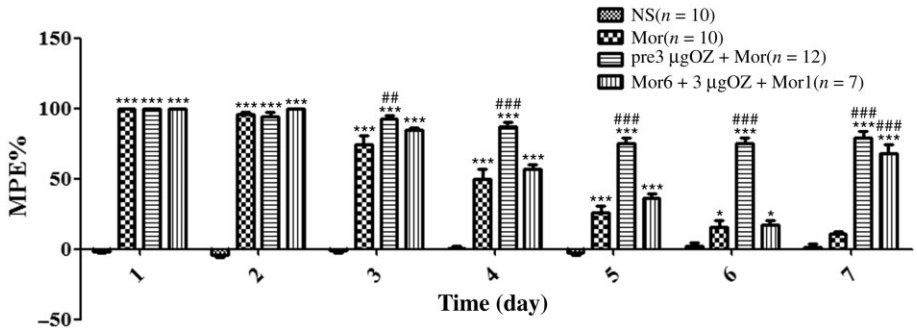

Figure 6

Bolus intrathecal injection of TAK1 inhibitor prevents or reverses morphine-induced antinociceptive tolerance. Bolus injection of OZ 24 h before the first morphine injection caused retention of the morphine efficacy and prevented the development of antinociceptive tolerance. Bolus injection of OZ on day 6 restored the morphine-induced antinociception on day 7. $^{*}P < 0.05$, $^{***}P < 0.001$, significantly different from control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significantly different from morphine group; two-way ANOVA.

reversed the development of morphine antinociceptive tolerance. All the data from this work support the idea that inhibition of TAK1 partly relieves the development of tolerance to repeated morphine-induced antinociception, and this might be due to the modulation by TAK1 of the MAPK members, p38 and/or JNK.

TAK1, a member of the MAPK kinase kinase family, has been suggested to contribute to the mechanical hypersensitivity after nerve injury when induced in spinal astrocytes (Katsura *et al.*, 2008). A study by Klatt and colleagues also suggested that the therapeutic inhibition of TAK1 might be used to reduce pain and relieve inflammatory symptoms (Klatt *et al.*, 2010). Evidence has emerged that opioid tolerance and pathological pain may share common cellular mechanisms (Mao *et al.*, 1995b; Mayer *et al.*, 1999). Therefore, this project was designed to explore the involvement of TAK1 in the development of morphine-induced antinociceptive tolerance in the spinal cord. Here, we demonstrated that chronic morphine exposure increased the expression of total TAK1 and p-TAK1, suggesting that the development of morphine tolerance is mediated not only through the activation of TAK1 (*via* phosphorylation), but also through a positive feedback cycle by which morphine-induced activated TAK1 drives greater TAK1 expression. TAK1 induced in spinal astrocytes has been shown to contribute to the mechanical hypersensitivity after nerve injury (Katsura *et al.*, 2008), and Goldmann *et al.* suggested that TAK1 is pivotal in CNS autoimmunity (Goldmann *et al.*, 2013). Here, we showed for the first time that TAK1 activation in spinal neurons occurs after chronic morphine exposure. The double-immunostaining experiments revealed that TAK1 was mainly co-localized with NeuN, a marker of neurons, and less or not at all with the markers of microglia and astrocytes.

Activation of TAK1 *via* different pathways may lead to differences in localization: after activation by non-canonical wnt signalling, TAK1 translocates to the nucleus, whereas the TAK1 activated in response to bone morphogenetic proteins apparently acts predominantly in the cytoplasm. Here, we showed that activated TAK1 receptors after chronic morphine exposure were clearly localized in clusters on the nucleus by co-staining with the nucleus-specific marker DAPI. Based on these findings, non-canonical wnt signalling may play a critical role in morphine-induced TAK1 activation. The role of the non-canonical wnt/Ca^{2+} pathway is known to help regulate Ca^{2+} release from the endoplasmic reticulum to control intracellular Ca^{2+} levels. Activation of TAK1 in the development of morphine antinociceptive tolerance may result from an imbalance of intracellular Ca^{2+}, as we have discussed previously (Xu *et al.*, 2007; 2008).

The natural compound (5Z)-7-oxozeaenol (OZ), a sorcylic lactone of fungal origin, was first shown by Tsuji *et al.* to be a selective TAK1 inhibitor, which acts by irreversibly inhibiting the binding of ATP to TAK1 (Ninomiya-Tsuji *et al.*, 2003). Since then, OZ has been widely studied in inflammation-related diseases, cancer, cerebral ischaemia and traumatic brain injury (Omori *et al.*, 2010; Neubert *et al.*, 2011; Sakurai, 2012; Zhang *et al.*, 2013). We confirmed here that the increased expression of TAK1 after chronic morphine exposure was inhibited by OZ *in vitro* and *in vivo*, while OZ prevented the development of tolerance to morphine-induced antinociception *in vivo*. Taken together, we conclude that morphine treatment might increase TAK1 enzymic activity, which leads to primary tolerance to morphine in rats; the increased TAK1 protein expression and phosphorylation then result in a changed signal transduction, which might lead to total tolerance to morphine-induced antinociception in rats. OZ might function in blocking the vicious circle by inhibiting enzymatic activity, and eventually attenuating morphine tolerance. But how does TAK1 activation result in tolerance to morphine in rats?

TAK1 has been reported to serve as a common upstream kinase of p38 MAPK and JNK *via* activating MAPKKs including MKK3/6 (Moriguchi *et al.*, 1996b; Shirakabe *et al.*, 1997) and MKK4/7 (Yamaguchi *et al.*, 1995). Accumulating evidence has suggested that activation of JNK and p38 MAPK in the spinal dorsal horn is involved in the induction and maintenance of morphine antinociceptive tolerance (Ma *et al.*, 2001; Cui *et al.*, 2006; Horvath *et al.*, 2010). p38 MAPK, a transducer of various extracellular stimuli, regulates the release of inflammatory factors (Saklatvala, 2004) and is involved in the development of morphine antinociceptive tolerance. The results of the present study are consistent with

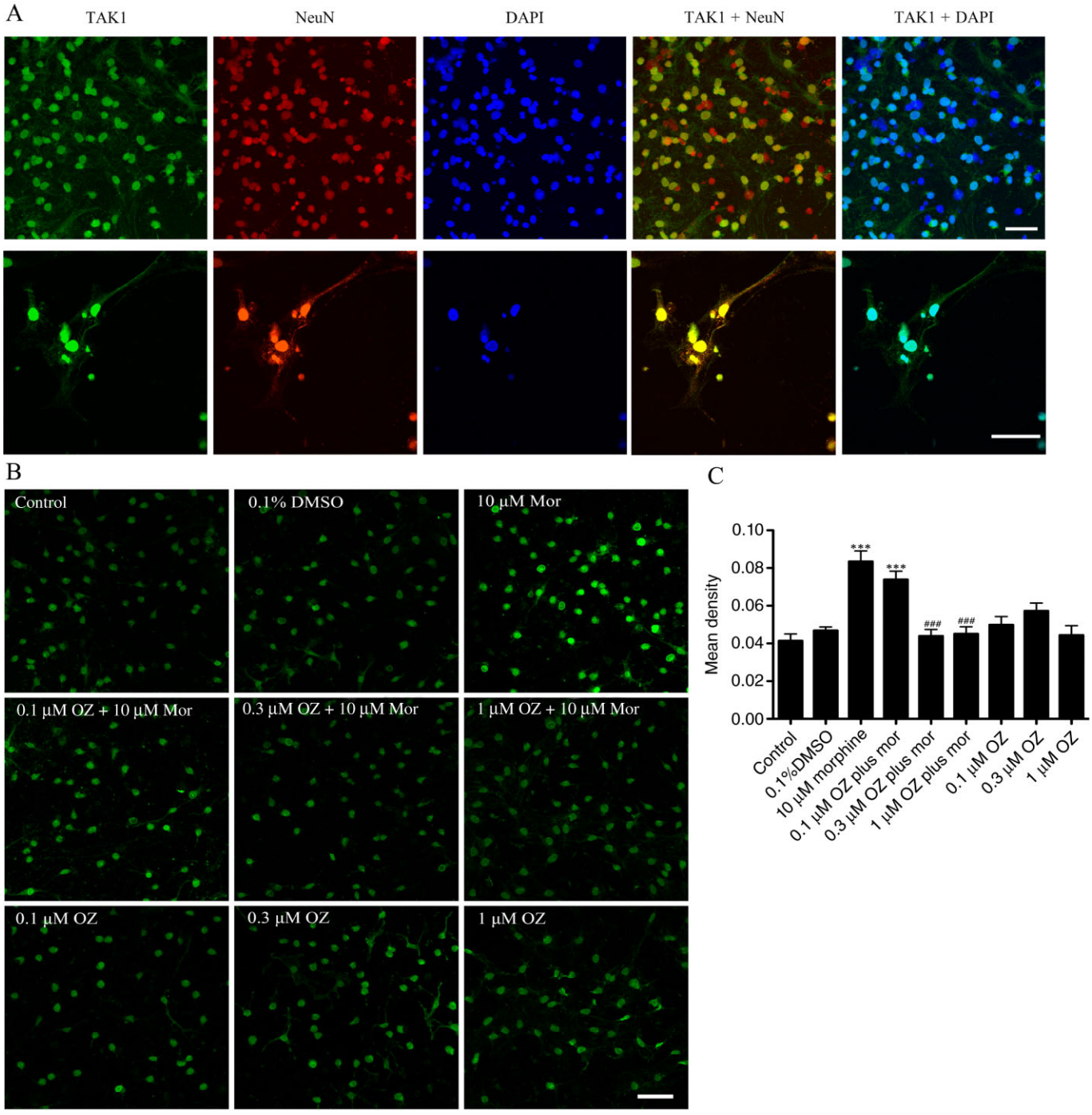

Figure 7

OZ reduced TAK1 expression after chronic morphine exposure in primary neuronal cell cultures. (A) Confocal photomicrographs showing a high degree of co-localization of TAK1 (green) with NeuN (red) in cultured neurons; TAK1 was mainly expressed in the nuclei stained with DAPI. (B and C) Chronic morphine treatment (10 μM) increased the mean fluorescence density of TAK1-positive cells, and this was reversed by co-administration of 0.3 and 1 μM OZ, but not 0.1 μM OZ. ***$P < 0.001$, significantly different from control group; ###$P < 0.001$, significantly different from morphine group; one-way ANOVA. Density, total integral optical density/area of cells.

previous studies (Horvath *et al.*, 2010; Wang *et al.*, 2010b) showing that chronic morphine exposure results in a remarkable increase in p38 MAPK phosphorylation, and this activation leads to the production of inflammatory mediators that further sensitize dorsal horn neurons (DeLeo and Yezierski, 2001; Watkins *et al.*, 2001). Meanwhile, repeated morphine treatment enhances the release of proinflammatory cytokines (Johnston *et al.*, 2004; Merighi *et al.*, 2012), and antagonists

of these cytokines and chemokines potentiate acute morphine analgesia and attenuate the development of morphine analgesic tolerance (Johnston *et al.*, 2004). Hence, the p38 MAPK signalling cascade might contribute to the development of morphine antinociceptive tolerance by regulating the levels of inflammatory factors. Moreover, p38 MAPK activation was demonstrated to trigger μ-opioid receptor endocytosis *via* small GTPase Rab5 (Mace *et al.*, 2005), indicating a vital role of p38 MAPK in the development of morphine tolerance. In addition to p38 MAPK, JNK has also been reported to be involved in the development of morphine analgesic tolerance (Ma *et al.*, 2001). Repeated morphine exposure increases the number of neurons expressing phosphorylated JNK and results in spinal JNK activation (Ma *et al.*, 2001; Fan *et al.*, 2003; Cui *et al.*, 2008; Guo *et al.*, 2009). The TRPV1 cation channel which is crucial for the transduction of noxious stimuli, mediates morphine tolerance *via* the activation of both p38 MAPK and JNK (Chen *et al.*, 2008). Consistent with previous studies, our results showed that chronic morphine exposure led to a remarkable increase in p38 MAPK and JNK phosphorylation. And pharmacological inhibition of TAK1 by OZ suppressed these increases, suggesting that p38 MAPK and JNK may be the downstream effectors of TAK1 activation in the development of morphine analgesic tolerance in the spinal cord. Based on the findings, we conclude that chronic morphine induces p38 and JNK activation in the spinal cord *via* TAK1 activation, and that the activation of the TAK1/JNK and/or TAK1/p38 signalling cascade contributes to the development of morphine antinociceptive tolerance.

Another indispensible member of the MAPK family, ERK, also participates in the development of morphine tolerance (Ma *et al.*, 2001; Wang *et al.*, 2009). Pathak *et al.* recently demonstrated that TAK1 may activate ERK *via* MEK1/2 (Pathak *et al.*, 2012). Hence, further work is needed to confirm whether the TAK1/ERK pathway takes part in the development of tolerance to morphine analgesia. In addition, the TAK1/TAB1/NIK cascade is involved in cytokine suppression of adipogenesis and PPAR-γ function (Suzawa *et al.*, 2003). PPAR-γ has recently been shown to play a tonic role in the modulation of morphine tolerance, and its pharmacological activation may help to reduce this (Ghavimi *et al.*, 2014; de Guglielmo *et al.*, 2014). Besides, TAK1 has emerged as a key regulator of signal transduction cascades leading to the activation of the transcription factor NF-κB (Adhikari *et al.*, 2007). Chronic morphine exposure induces a pronounced increase in NF-κB activation (Wang *et al.*, 2010a), and down-regulating the activation of the canonical NF-κB pathway blocks astrocytic and microglial cell activation, and suppresses the increase in TNF-α, IL-1β and IL-6 in the dorsal horn, thus blocking antinociceptive tolerance (Ndengele *et al.*, 2009). Hence, there is a possibility of the involvement of TAK1-dependent NF-κB activation in morphine-induced antinociceptive tolerance. Accordingly, further studies are needed to confirm whether TAK1 up-regulation induces the activation of NF-κB in the dorsal horn and affects the morphine-induced antinociception.

Furthermore, a previous study showed that OZ penetrates the blood–brain barrier (Ninomiya-Tsuji *et al.*, 2003). Thus, it will be interesting to explore the effect of OZ administered systemically, as drugs are used in clinical therapy. As such, TAK1 inhibition may provide a clinical paradigm for preventing morphine antinociceptive tolerance. A TAK1 inhibitor such as OZ could be a therapeutic pharmacological tool, alone or in combination with other agents (e.g., NMDA receptor antagonists), to prevent the development of tolerance.

In summary, the present study established TAK1 as a crucial molecule in the development of morphine antinociceptive tolerance. Modulation of TAK1 activation by a selective inhibitor in the lumbar spinal cord may prove an attractive target for adjuvant therapy to attenuate such tolerance. These findings highlight the possibility of a new clinical strategy to prevent morphine antinociceptive tolerance.

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Conflicts of interest

The authors declare no conflicts.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Mechanonociceptive data (g) for the Randall–Sellitto tests of Figure 1A.
Table S2 Mechanonociceptive data (g) for the Randall–Sellitto tests of Figure 3.
Table S3 Mechanonociceptive data (g) for the Randall–Sellitto tests of Figure 6.