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ORIGINAL ARTICLE

Resveratrol reverses morphine-induced neuroinflammation in morphine-tolerant rats by reversal HDAC1 expression

Ru-Yin Tsai [a], Juei-Chii Wang [b], Kuang-Yi Chou [c], Chih-Shung Wong [b,d,e,g,**], Chen-Hwan Cherng [f,*]

[a] College of Nursing and Health Sciences, Da-Yeh University, Changhua, Taiwan
[b] Department of Anesthesiology, Cathay General Hospital, Taipei, Taiwan
[c] General Education Center, National Taipei University of Nursing and Health Sciences, Taipei, Taiwan
[d] School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan
[e] Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan
[f] Department of Anesthesiology, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan

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Background/purpose: We previously showed that subsequent intrathecal (i.t.) injection of resveratrol (30 μg) significantly reverses morphine-evoked neuroinflammation in morphine-tolerant rats. The present study examined the underlying mechanism.
Methods: Male Wistar rats were implanted with two i.t. catheters, one of which was connected to a miniosmotic pump and used for morphine (15 μg/h) or saline infusion for 120 hours. To examine the effects on spinal cord expression of histone deacetylase 1 (HDAC1), the inflammatory cytokine tumor necrosis factor-α (TNF-α), and TNF receptor (TNFR) 1 and TNFR2 during tolerance induction, a tail-flick test was performed prior to infusion and after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of infusion.
Results: Resveratrol treatment prior to morphine challenge restored the antinociceptive effect of morphine in morphine-tolerant rats and reversed the morphine infusion-induced increase in HDAC1, TNF-α, and TNFR1 expression. Moreover, chronic morphine infusion increased TNFR1-specific expression in neuron in morphine-tolerant rat spinal cords, and this effect was almost completely inhibited by resveratrol treatment prior to morphine challenge.
Conclusion: Resveratrol restores the antinociceptive effect of morphine by reversing morphine infusion-induced spinal cord neuroinflammation and increase in TNFR1 expression. The

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* Corresponding author. Department of Anesthesiology, Tri-Service General Hospital, Number 325, Chenggung Road, Section 2, Neihu 114, Taipei, Taiwan.
** Corresponding author. Cathay General Hospital, 280, Renai Road, Section 4, Taipei, Taiwan.
E-mail addresses: w82556@gmail.com (C.-S. Wong), cherng1018@yahoo.com.tw (C.-H. Cherng).
[g] Equal contribution as a corresponding author.

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reversal of the morphine-induced increase in TNFR1 expression by resveratrol is partially due to reversal of the morphine infusion-induced increase in HDAC1 expression. Resveratrol pretreatment can be used as an adjuvant in clinical pain management for patients who need long-term morphine treatment or with neuropathic pain.

Introduction

Morphine is a powerful analgesic for treating severe pain, but its long-term administration induces tolerance, which hampers its clinical use. Morphine tolerance is a complex physiologic process,[1] and various mechanisms have been proposed, such as glutamatergic receptor activation[2] and neuroinflammation.[3,4] The opioid-induced hyperalgesia observed in tolerant rats[5] is similar to the symptoms observed in neuropathic pain patients, in whom opioids provide only a limited analgesic effect. Recent studies have suggested that expression of pain-associated genes in sensory neurons and glial cells is involved in the generation and maintenance of neuropathic pain[6,7] and morphine tolerance.[8] Epigenetic processes induce heritable changes in gene expression and function and regulate the transcription and expression of pro- or antinociceptive genes after chronic morphine exposure.[8,9] Epigenetic modifications influence the inflammatory process,[10] and an inhibitor of histone deacetylase (HDAC) decreases expression of cytokines, especially tumor necrosis factor-alpha (TNF-α).[11] However, the contribution of histone acetylation to the development of morphine tolerance is not clear. Binding of inflammatory cytokines to their receptors may modulate epigenetic processing. In the central nervous system, TNF-α, which is constitutively expressed in neurons and glial cells and is released from these cells on stimulation,[12] plays a crucial role in the glial—neuron interaction that affects both neuropathic pain[13] and drug abuse.[14] TNF inhibitors are widely used for the treatment of inflammatory diseases. Our recent studies showed that the binding of TNF-α to TNF receptors (TNFRs) modulates TNF-α-mediated inflammatory signal transduction.[2,15]

Polyphenolic compounds are found in many plants, and one of these compounds, resveratrol, has been shown to have beneficial effects as a neuroprotective,[16] anticancer, and anti-inflammation agent.[17] These results imply that resveratrol may have an analgesic activity. Resveratrol has been shown to inhibit nitric oxide generation and TNF-α expression in diabetic rats and spinal nerve-ligated rats.[16,18] In our previous study, resveratrol reversed the morphine infusion-induced increase in expression of the proinflammatory cytokines TNF-α, interleukin (IL)-1β, and IL-6 and microglia activation.[4] These results suggest that resveratrol has the potential for relieving pain in patients who develop morphine tolerance. The mechanism by which acute resveratrol treatment inhibits neuroinflammation after development of morphine tolerance remains unclear. The present study is the first to examine this mechanism using morphine-evoked neuroinflammation in morphine-tolerant rats, and showed that long-term morphine infusion resulted in increased expression of TNF-α and TNFR1 and that these effects were reversed by resveratrol, possibly by reversing the morphine infusion-induced increase in HDAC1 expression seen in the tolerant rat spinal cord.

Methods

Construction of the intrathecal catheters

Each intrathecal (i.t.) catheter was constructed by inserting a 3.5-cm silastic tube (Dow Corning, Midland, MI, USA) into an 8-cm polyethylene tube (0.008 inch inner diameter, 0.014 inch outer diameter; Spectranetics, Colorado Springs, CO, USA) and sealing the joint with epoxy resin and silicon rubber.

Animal preparation, i.t. drug delivery, and sample collection

The use of rats in this study was reviewed and approved by the National Defense Medical Center Animal Care and Use Committee, Taipei, Taiwan. Male Wistar rats (350—400 g; Biolasco Taiwan Co., Taipei, Taiwan) underwent a tail-flick test (preinfusion test) and, on the following day, they were anesthetized with phenobarbital (65 mg/kg, intraperitoneally). Two i.t. catheters were implanted via the atlanto-occipital membrane down to spinal cord segments L5—S3, which control the tail flick reflex.[19] Next, the rats were returned to their home cages, with each rat being housed individually and maintained on a 12-hour light/dark cycle with food and water freely available. Rats with neurological deficits were excluded.

To examine the effects of morphine tolerance on spinal cord expression of HDAC1, TNF-α, and TNFR1 and TNFR2, one of the catheters was connected to a miniosmotic pump (Alzet, Cupertino, CA, USA), and either morphine (Sigma—Aldrich, St. Louis, MO, USA; 15 μg/h) or saline was infused for 120 hours at a rate of 1 μL/h. A tail-flick test was performed prior to catheter insertion and after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of infusion. Then rats were sacrificed after 24 hours, 72 hours, and 120 hours of morphine infusion by exsanguination under anesthesia with isoflurane (Abbott Laboratories Ltd., Queenborough, Kent, UK). The lumbar enlargement of the spinal cord was removed to measure mRNA and protein expression, as described in a later section.

To examine the effects of resveratrol on morphine tolerance and protein expression, infusion was discontinued after 120 hours. After an interval of 3 hours, the second catheter was used to inject rats i.t. with 5 μL of

either resveratrol [Sigma; 30 μg in dimethyl sulfoxide (DMSO)] or DMSO, then, after 30 minutes, with 15 μg of morphine (5 μL in saline). The rats were then subjected to a nociceptive tail-flick test every 30 minutes for the next 120 minutes. Later, the lumbar enlargement of the spinal cord was removed for histochemical analysis and to measure protein expression as described below. After each drug injection, the catheter was flushed with 8 μL saline (dead volume of the catheter). No abnormal motor function was observed after injection of the test drugs.

Antinociceptive test

The tail-flick latency was measured using the hot water immersion test (52 ± 0.5°C). The baseline latency was approximately 2 ± 0.25 seconds, and a 10-second cutoff time was used. The tail-flick latency was used to measure the antinociceptive effect of morphine as described previously.[20,21] The rats were placed in plastic restrainers for drug injection and antinociception assessment.

Spinal cord sample collection and membrane fraction preparation

To collect samples of spinal cord, the rats were sacrificed by exsanguination under isoflurane anesthesia. Laminectomy was performed at the lower edge of the 12th thoracic vertebra, and the lumbar enlargement of the spinal cord immediately removed. Part of the sample was embedded in optimal cutting temperature compound (Sakura Finetec Inc., Torrance, CA, USA) for histochemistry, whereas the dorsal region of the remainder was removed and was either stored at −80°C until used for Western blotting, cytokine analysis, or real-time polymerase chain reaction (PCR) or used immediately to prepare the membrane fraction using a cytoplasmic, nuclear, and membrane compartment protein extraction kit as recommended by the manufacturer (Biochain Institute, Inc., Hayward, CA, USA). The membrane fraction was checked for specificity by Western blotting, as described in the next section, using mouse antibodies against rat Na^+/K^+ ATPase, and then stored at −80°C until analysis for TNFR1 and TNFR2 expression.

Western blots

Whole homogenate

The supernatant prepared from a homogenate of the dorsal portion of the lumbar spinal cord in the same way as for measurement of TNF-α levels was used for Western blotting. Samples containing 15 μg of protein were adjusted to a similar volume in loading buffer [10% sodium dodecyl sulfate (SDS), 20% glycerin, 125mM Tris, 1mM EDTA, 0.002% bromphenol blue, 10% β-mercaptoethanol] and denatured at 95°C for 10 minutes. Then they were separated on a 10% SDS-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was blocked for 1 hour at room temperature in blocking buffer (5% nonfat milk in 50mM Tris—HCl, 154mM NaCl, and 0.05% Tween 20; pH 7.4) and incubated overnight at 4°C with polyclonal rabbit antirat HDAC1 antibodies (Cell Signaling Technology Inc., Danvers, MA, USA; 1:2500 in blocking buffer), then for 1 hour at room temperature with horseradish peroxidase-conjugated donkey antirabbit immunoglobulin G antibodies (Chemicon, Temecula, CA, USA; 1:5000 in blocking buffer). Bound secondary antibodies were then detected using Chemiluminescence^{plus} reagent (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) and a chemiluminescence imaging system (Syngene, Cambridge, UK), and the optical density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match Software, Syngene, Cambridge, UK). Finally, the blots were incubated at 56°C for 18 minutes in stripping buffer (0.0626 mol/L Tris—HCl, pH 6.7, 2% SDS, 0.1 mol/L mercaptoethanol) and reprobed with a monoclonal mouse antihistone H1 antibody (Abcam, Naka-Ku Nagoya, Japan) as the loading control.

Membrane proteins

The membrane fraction was checked for specificity by Western blotting as described above using a 1:2000 dilution in blocking buffer of mouse antibodies against rat Na^+/K^+ ATPase (Abcam, Naka-Ku Nagoya, Japan). Analysis of membrane TNFR1 and TNFR2 protein levels was performed by Western blotting as described above using polyclonal rabbit antibodies against rat TNFR1 or TNFR2 (both from Abcam, Cambridge Science Park, Cambridge, UK).

Measurement of TNF-α levels

The dorsal portion of the lumbar spinal cord was homogenized at 4°C in RIPA lysis buffer (Upstate, USA, Inc., Charlottesville, VA, USA); the homogenate was then centrifuged at 10,000*g* for 30 minutes at 4°C and the supernatant used for assays. The protein concentration of samples was determined using the bicinchoninic acid assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) with bovine serum albumin as the standard. The TNF-α level in the supernatant was measured with a simultaneous multiplexed format and a microbead and flow-based protein detection system (Bio-Plex Suspension Array System; Bio-Rad Laboratories Inc.) based on Luminex xMAP technology. Each sample was adjusted to a protein concentration of 1 μg/μL in the Bio-Plex kit lysis buffer, and the lysate was incubated with antirat TNF-α antibody-coated fluorescence-coded microbeads for 90 minutes at room temperature; next, the beads were washed, incubated with biotinylated second antibodies for 30 minutes at room temperature, washed again, incubated with a streptavidin-coupled phycoerythrin reporter system at room temperature for 10 minutes, washed, and finally analyzed by flow cytometry. All spinal cord samples were assayed in triplicate, and the TNF-α concentration was determined using a recombinant mouse TNF-α internal standard curve. The coefficient of variation for controls ranged from 9% to 19% on the exponential phase of the standard curve.

Immunocytochemistry and image analysis

The lumbar enlargement (L5—S3) of the spinal cord was embedded in optimal cutting temperature compound (Sakura Finetec Inc., USA), then frozen sections (5 μm) were prepared and fixed by immersion in ice-cold acetone/methanol (1:1) for 5 minutes. After a wash in ice-cold

phosphate-buffered saline, the sections were blocked by incubation for 1 hour at room temperature in blocking buffer (phosphate-buffered saline/10% normal goat *serum*/0.1% Triton X-100), then were double-labeled by overnight incubation at 4°C with unlabeled polyclonal rabbit antirat TNFR1 antibody and fluorescein isothiocyanate-labeled mouse monoclonal antibody against rat CD11b/c (OX42; microglial cell marker; Serotec, Oxford, UK), rat GFAP (astrocyte marker; Molecular Probe, Eugene, OR, USA), or NeuN (neuronal nuclei marker; Chemicon, USA), then for 1 hour at room temperature with rhodamine-labeled goat antirabbit immunoglobulin G antibodies (Abcam) in blocking buffer. Images were then captured using an Olympus BX 50 fluorescence microscope (Olympus, Optical, Tokyo, Japan) and a Delta Vision disconsolation microscopic system operated by SPOT software (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Controls without primary antibody were run to confirm the specificity of staining.

Quantitative real-time PCR

Total RNA was extracted from the dorsal portion of the lumbar spinal cord by homogenization of the sample at 4°C in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA synthesis reaction was carried out using 1 μg of total RNA, 50 ng of random hexamer primer, 0.5mM deoxynucleotide mix, 10mM dithiothreitol, 1× RT buffer, and 200 U of Superscript III reverse transcriptase (Invitrogen) in a total volume of 20 μL, using conditions of 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 5 minutes. Real-time PCR was performed using an Applied Biosystems Prism 7500 Sequence Detection system with FastStart Universal SYBR Green Master mix (ROX; Roche Applied Science, Mannheim, Germany), gene-specific primers, and diluted cDNA, using thermal cycle conditions of 10 minutes at 95°C, two-step PCR for 40 cycles of 95°C/15 seconds, and a final incubation at 60°C for 1 minute. Fluorescence data were acquired during each extension phase. After 40 cycles, a melting curve was generated to verify primer specificities. All samples were tested in triplicate. The amplification data were analyzed using Applied Biosystems Prism Sequence Detection Software version 1.1 (Roche Applied Science). To compare the relative expression levels after different treatments, the expression of the gene of interest was normalized to that of the GAPDH control using the $\Delta\Delta C_T$ method recommended by the manufacturer. The primer sequences were TNF-α (NM_012675; forward: 5′-CACCGGCAAGGATTCCAA-3′, reverse: 5′-CACTCAGGCATCGACATTCG-3′), HDAC1 (NM_001025409.1; forward: 5′-TGCTGGACTTACGAGACAGC-3′, reverse: 5′-GGAAGGGCTGATGTGAAGC-3′), TNFR1 (NM_013091.1; forward: 5′-AATGAGTGCACCCCTTGC-3′, reverse: 5′-CCTGGGGGTTTGTGACATT-3′), and TNFR2 (NM_130426.4; forward: 5′-GAGGCCCAAGGGTCTCAG-3′, reverse: 5′-GCTGCCATGGGAAGAATC-3′).

Statistical analysis

All data are presented as the mean ± standard error of the mean. Statistical analysis was performed using SigmaStat 3.0 software (SYSTAT Software Inc., San Jose, CA, USA). Differences in tail-flick latency were analyzed using two-way analysis of variance (ANOVA; time and treatment), followed by one-way ANOVA (at each time of the experiment) with a *post hoc* Student–Newman–Keuls test. To compare immunoblotting results, the intensity of each test band was expressed as the optical density relative to that of the average optical density for the corresponding control band, and the data were analyzed using one-way ANOVA, followed by multiple comparisons with the Student–Newman–Keuls *post hoc* test. A significant difference was defined as $p < 0.05$.

Results

Effect of chronic morphine infusion on HDAC1 levels in spinal cord dorsal horn

As shown in Figure 1, i.t. infusion of saline for 120 hours did not have any antinociceptive effect, as measured by the tail-flick test (2.0 ± 0.23 seconds). By contrast, in rats infused for 120 hours with morphine (15 μg/h), the maximum antinociceptive effect was seen after 24 hours (9.12 ± 0.18 s; $p < 0.0001$), and tolerance was first seen at 48 hours (5.73 ± 0.34 s; $p < 0.0001$) and became maximal at 72–120 hours (2.82 ± 0.51 seconds at 72 hours, $p = 0.0056$; 2.36 ± 0.3 seconds at 96 hours, $p = 0.0731$; and 2.22 ± 0.22 seconds at 120 hours, $p = 0.1078$). As shown by immunoblotting of the supernatants from spinal cord dorsal horn, tolerance was associated with a significant time-dependent increase in HDAC1 protein levels (Figures 2B and 2C; $p = 0.374$, $p < 0.001$, $p < 0.001$), but not mRNA levels (Figure 2A).

Effect of morphine infusion on TNF-α and TNFR levels

As in our previous study,[4] a significant increase in TNF-α expression was seen in the dorsal spinal cord during

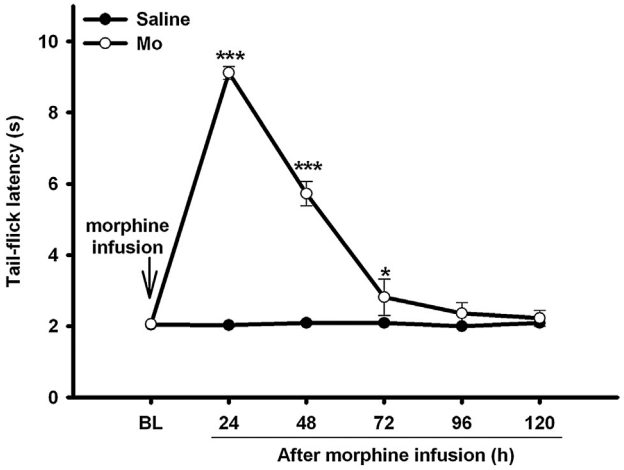

Figure 1 Effect of morphine infusion on thermal hyperalgesia. Time-course of changes in the tail-flick latency after 24 hours, 48 hours, 72 hours, 96 hours, or 120 hours of continuous intrathecal infusion of saline (1 μL/h) or morphine (15 μg/h; 1 μL/h). All data are expressed as the mean ± standard error of the mean (SEM) for five rats. *$p < 0.01$, ***$p < 0.0001$ compared to the saline-infused group ($n = 5$ of each group). BL = baseline; Mo = morphine.

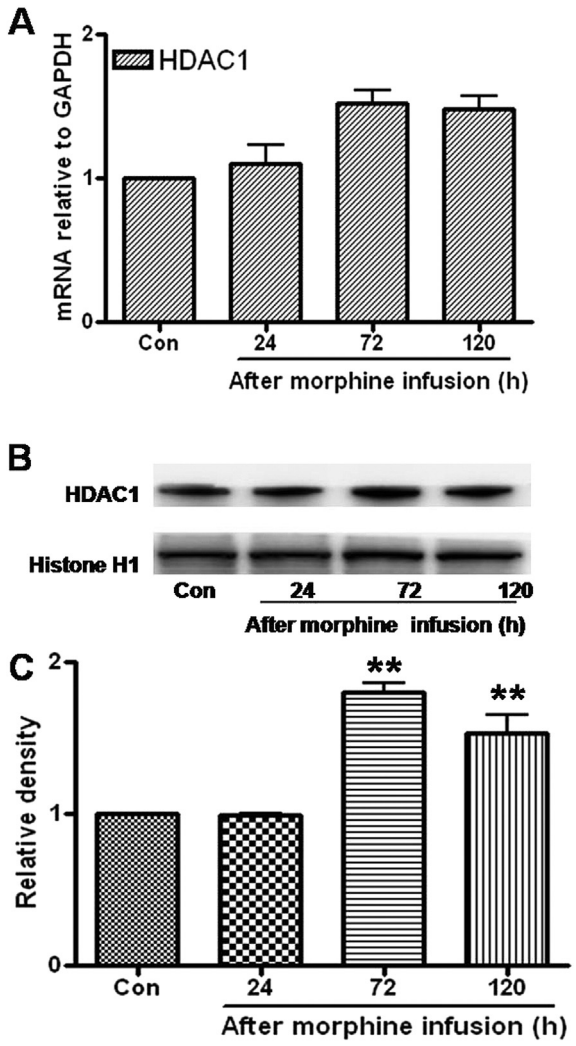

Figure 2 Time-course of changes in histone deacetylase 1 (HDAC1) levels in rat spinal cord dorsal horn during morphine infusion. (A) HDAC1 mRNA and (B) protein levels were measured in lumbar spinal cord dorsal horn lysate from rats infused with saline (Con) or rats infused with morphine for 24 hours, 72 hours, and 120 hours. (C) Quantification of the data from (B) for at least three rats (mean ± standard error of the mean). $^{**}p < 0.001$ compared to the saline-infused control group.

tolerance development in terms of both mRNA (Figure 3A; $p < 0.0001$ at 24 hours, 72 hours, and 120 hours) and protein (Figure 3B; $p < 0.0001$ at 24 hours, 72 hours, and 120 hours). As shown in Figures 4A and 4B, levels of TNFR1 mRNA ($p = 0.085$ at 24 hours, $p < 0.0001$ at 72 hours and 120 hours) and TNFR2 mRNA ($p = 0.0038$ at 24 hours, $p < 0.0001$ at 72 hours, and $p = 0.0002$ at 120 hours) in the dorsal spinal cord also increased in a time-dependent manner. By contrast, although TNFR1 protein levels in the dorsal horn membrane fraction were increased at 24–120 hours ($p = 0.0001$ at 24 hours, $p = 0.0002$ at 72 hours, and $p = 0.0004$ at 120 hours), TNFR2 protein levels were not ($p = 0.724$ at 24 hours, $p = 0.711$ at 72 hours, and $p = 0.492$ at 120 hours; Figures 4C and 4D). These results suggest that TNF-α and TNFR1 play a critical role in the modulation of spinal synaptic transmission during both induction and maintenance of morphine tolerance.

We then examined the effect of i.t. injection of resveratrol 3 hours after the end of morphine infusion on the antinociceptive effect of morphine and on HDAC1, TNF-α, and TNFR1 expression.

Intrathecal resveratrol injection restores the antihyperalgesia effect of morphine in morphine-tolerant rats

As in our previous study,[4] when the rats were infused for 120 hours with saline or morphine as described above, then underwent challenge by i.t. injection of 15 μg of morphine 3.5 hours later, a significant antinociceptive effect (tail-flick latency) was seen in saline-infused rats (9.5 ± 0.35 seconds) but not in morphine-infused rats (2.0 ± 0.15 seconds; Figure 5). In addition, when the rats received an i.t injection of 30 μg of resveratrol in DMSO 30 minutes prior to morphine challenge, significant restoration of the antinociceptive effect of morphine was seen in morphine-tolerant rats, with a maximal effect at 60 minutes (6.28 ± 0.6 seconds, $p < 0.001$). This result showed that resveratrol pretreatment could partially restore the antinociceptive effect of morphine in morphine-tolerant rats.

Resveratrol reverses the increase in HDAC1 expression seen in the morphine-tolerant rat spinal cord

As shown in Figure 6, 120 hours of morphine infusion increased HDAC1 expression (2 ± 0.15-fold; $p < 0.005$) in the spinal cord lysate compared to rats infused with saline for 120 hours, and this effect was almost completely reversed by resveratrol treatment 3 hours after the end of infusion (1.13 ± 0.12-fold; $p = 0.33$).

Resveratrol reverses the increase in proinflammatory cytokine TNF-α levels seen in the morphine-tolerant rat spinal cord dorsal horn

TNF-α levels in the rat spinal cord dorsal horn lysate were measured using Bio-Plex cytokine assay kits. As shown in Figure 7, TNF-α levels were significantly higher in morphine-infused/DMSO-injected rats (57.66 ± 2.74 pg/mg; $p < 0.0001$) than in saline-infused/DMSO-injected rats (5.0 ± 0.68 pg/mg), and this effect was considerably reversed by i.t. injection of resveratrol 3 hours after the end of morphine infusion (20.83 ± 3.09 pg/mg; $p < 0.0001$ compared to morphine-infused/DMSO-injected rats).

Resveratrol reverses the morphine-evoked increase in TNFR1 expression in spinal cord dorsal horn cell membranes and in activated glial cells

Figure 8 shows Western blots of TNFR1 levels in cell membranes from the various groups, with the value for the saline-infused/DMSO-injected group set as 1. TNFR1 levels were significantly higher in morphine-infused/DMSO-injected rats (2.73 ± 0.74; $p < 0.0001$) than in saline-

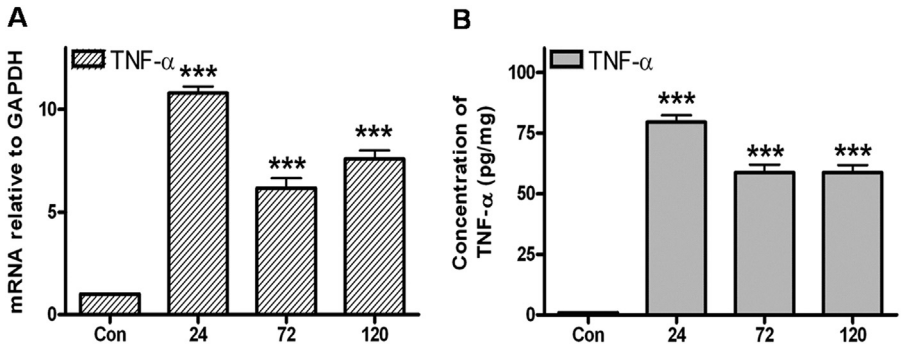

Figure 3 Time-course of changes in tumor necrosis factor-alpha (TNF-α) levels in rat spinal cord dorsal horn after morphine infusion. (A) TNF-α mRNA and (B) protein levels were measured in lumbar spinal cord dorsal horn from rats infused with saline (Con) or rats infused with morphine for 24 hours, 72 hours, and 120 hours. All data are expressed as the mean ± standard error of the mean for three rats. ***$p < 0.0001$ compared to the saline-infused group.

infused/DMSO-injected rats, and this effect was completely reversed by i.t. injection of resveratrol 3 hours after the end of morphine infusion (0.72 ± 0.03; $p < 0.0001$).

Figure 9 shows the results of a double-staining immunohistochemical study, which demonstrate that, in the spinal cord dorsal horn of morphine-tolerant rats (Mo + DMSO), the morphology of astrocytes (GFAP) and microglia (OX42), but not neurons (NeuN), changed from a ramified shape to an ameboid shape, showing the activation of these cells in the morphine-tolerant rat spinal cor. In addition, strong TNFR1 immunoreactivity was seen in neurons (white arrows), but not in glial cells, in the same rats. Again, both effects were reversed by resveratrol treatment 3 hours after the end of morphine infusion. These results support the idea that activation of TNFR1 signaling in the morphine-tolerant rat spinal cord might play an important role in the development of the thermal hyperalgesia seen in morphine-tolerant rats.

Discussion

Resveratrol has been widely studied in the treatment of inflammatory disorders.[16,22] A pilot study showed that intraperitoneal injection of resveratrol dramatically improves the antinociceptive effect in carrageenan-evoked hyperalgesia rats.[23] Similarly, our study demonstrated that i.t. injection of resveratrol restored the

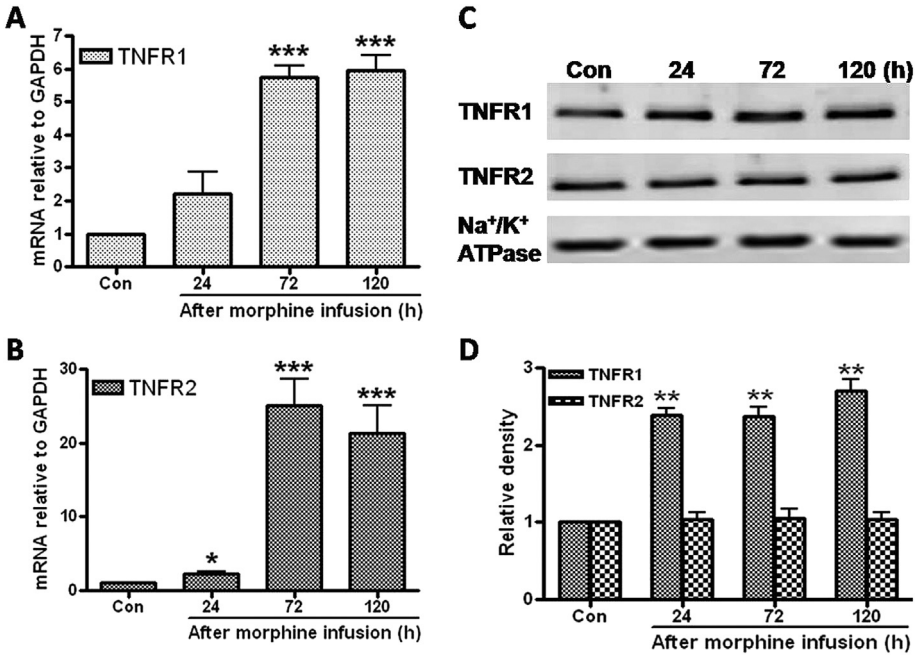

Figure 4 Effects of morphine infusion on tumor necrosis factor receptor 1 (TNFR1) and 2 (TNFR2) mRNA levels in rat spinal cord dorsal horn and protein levels in membranes. Time-course of changes in (A) TNFR1 and (B) TNFR2 mRNA levels in spinal cord dorsal horn and corresponding protein levels in (C and D) membranes from rats infused with saline (Con) or rats infused with morphine for 24, 72, and 120 hours. All data are expressed as the mean ± standard error of the mean for three rats. **$p < 0.001$, ***$p < 0.0001$ compared to the saline-infused group.

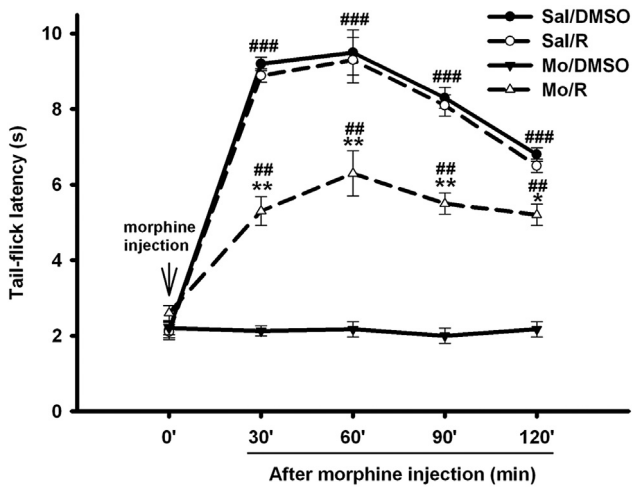

Figure 5 Resveratrol restores the antinociceptive effect of morphine in morphine-tolerant rats. At 3 hours after the end of 120 hours of morphine or saline infusion, the rats were injected with either dimethyl sulfoxide (DMSO; 5 μL) or resveratrol (30 μg in 5 μL of DMSO), then, 30 minutes later, underwent morphine challenge (15 μg in 5 μL of saline) and the tail-flick latency was measured every 30 minutes for 120 minutes. All data are expressed as the mean ± standard error of the mean for three rats. $^{*}p < 0.01$, $^{**}p < 0.001$ compared to the Sal/DMSO group. $^{\#\#}p < 0.001$; $^{\#\#\#}p < 0.0001$ compared to the Mo/DMSO group. Sal/DMSO = saline infusion plus DMSO injection; Sal/R = saline infusion plus resveratrol injection; Mo/DMSO = morphine infusion plus DMSO injection; Mo/R = morphine infusion plus resveratrol injection.

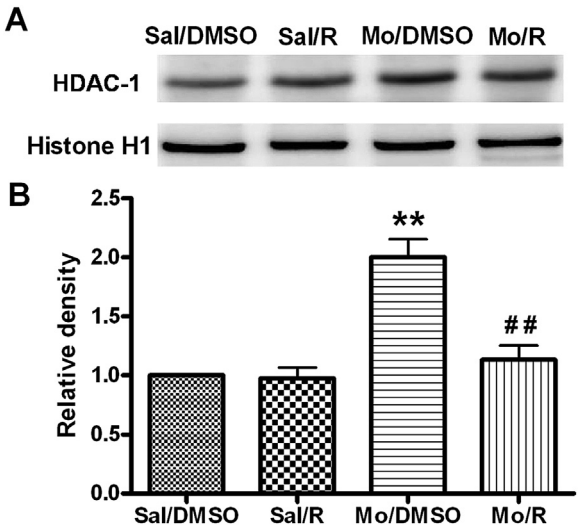

Figure 6 Resveratrol reverses the increase in histone deacetylase 1 (HDAC1) levels seen in the morphine-tolerant rat spinal cord. The infusion/injection protocol described in the legend to Figure 5 was used, then spinal cord dorsal horn were collected 120 minutes after morphine challenge. (A) Western blot analysis of HDAC1 levels in spinal cord dorsal horn from the indicated groups. (B) Quantification of the data from (A) expressed relative to the Sal + DMSO groups using histone 1 as the loading control. The data are the mean ± standard error of the mean for three rats per group. $^{**}p < 0.005$ compared to the Sal/DMSO group. $^{\#\#}p < 0.005$ compared to the morphine-tolerant group (Mo/DMSO). DMSO = dimethyl sulfoxide.

antinociceptive effect of morphine in morphine-tolerant rats and that this was associated with reversal of the morphine-induced increase in HDAC1, TNF-α, and TNFR1 levels and with reversal of astrocyte and microglia activation in the morphine-tolerant rat spinal cord.

Spinal cord glia activation seems to be a common mechanism of neuropathic pain and morphine tolerance.[24] The importance of neuroimmune activation in the initiation and facilitation of morphine tolerance has been demonstrated.[25] Long-term morphine administration leads to activation of calcitonin gene-related peptide (CGRP) receptors, which differentially control the synthesis and release of TNF-α by astrocytes and microglia via the p38 and ERK signaling pathways, and blockade of CGRP signaling in astrocytes and microglia has been shown to prevent TNF-α release and subsequent development of tolerance.[26] Previous studies have shown that chronic morphine treatment induces glia activation and expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the rat spinal cord.[27,28] The i.t. coadministration of morphine with a glia metabolic inhibitor[29] or a glial modulator[24] significantly attenuates morphine tolerance. Thus, limiting the production of proinflammatory cytokines by activated microglia and astrocytes should help reverse morphine tolerance. In this study, chronic morphine infusion induced significant increases in TNF-α mRNA and protein levels in the dorsal horn of the rat spinal cord, and the increase in TNF-α protein levels was almost completely reversed by subsequent i.t. injection of resveratrol. This suggests that resveratrol can regulate proinflammatory cytokine expression at both the transcriptional and translational levels by interacting with transcriptional factors (such as NFκp65 protein) and related signaling pathways.

The excitatory amino acid glutamate plays an important role in excitatory synaptic plasticity and morphine

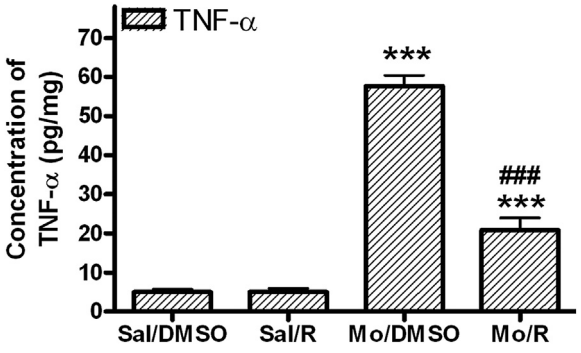

Figure 7 Resveratrol reverses the morphine-evoked increase in tumor necrosis factor-alpha (TNF-α) expression. The infusion/injection protocol described in the legend to Figure 5 was used, then spinal cord dorsal horn were collected 120 minutes after morphine challenge and tested for TNF-α levels. The results are expressed as the mean ± standard error of the mean, with three rats per group. $^{***}p < 0.0001$ compared to the Sal/DMSO group. $^{\#\#\#}p < 0.0001$ compared to the morphine-tolerant group (Mo/DMSO). DMSO = dimethyl sulfoxide.

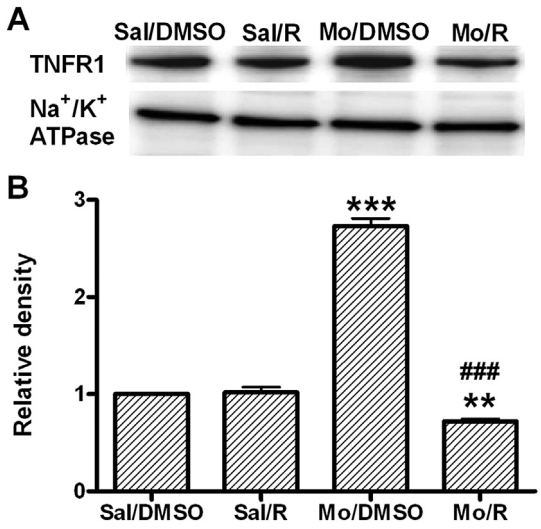

Figure 8 Resveratrol reverses the morphine-induced increase in TNFR1 expression. The infusion/injection protocol described in the legend to Figure 5 was used. (A) Western blot analysis of TNFR1 levels in the membrane fraction of the different treatment groups; Na^+/K^+ ATPase was used as the internal control. (B) Quantification of the data from (A) expressed relative to the Sal/DMSO value. The data are the mean ± standard error of the mean for three rats per group. $^{**}p < 0.001$; $^{***}p < 0.0001$ compared to the Sal/DMSO group. $^{\#\#\#}p < 0.0001$ compared to the morphine-tolerant group (M/DMSO). DMSO = dimethyl sulfoxide; TNFR1 = tumor necrosis factor receptor 1.

tolerance.[30] Synaptic glutamate levels are tightly controlled by transporters on neurons and astrocytes,[31] and the glutamate transporter GLT-1 is especially important in the rapid clearance of most synaptic glutamate in the central nervous system.[20] Enhanced nuclear factor kappa B (NF-κB) activity increases TNF-α expression, which, in turn, inhibits GLT-1 expression, leading to an increase in the synaptic glutamate concentration, resulting in cell toxicity and cell death in neuroinflammatory diseases.[32] Moreover, TNF-α can block glutamate reuptake via activated TNFR1, and this interaction between TNF-α and glutamate helps maintain glia/neuronal activation and further amplifies nociceptive signaling.[33] After nerve lesion, TNF-α levels are significantly increased in dorsal root ganglia neurons, and this is associated with thermal hyperalgesia and TNFR1 upregulation.[34,35] These results indicate that modulation of TNF-α and TNFR1 expression could be a biological target for the treatment of morphine tolerance. Our study supports the idea that reversal of morphine infusion-induced spinal neuroinflammation, and TNF-α and TNFR1 expression can help restore the antinociceptive effect of morphine after long-term morphine exposure. As per our recent studies and the present study, we found that morphine infusion induces glia cells to release TNF-α, but the TNFR1 was specifically expressed on neuron plasma membrane. These results suggest that TNF-α activates cytokine-mediated neuroinflammation in spinal glia cells and subsequently, via a paracrine way, induced TNFR1 expression in neurons. Although the role of TNF-α in morphine tolerance has been intensively studied, the temporal profile of TNF-α and TNFR

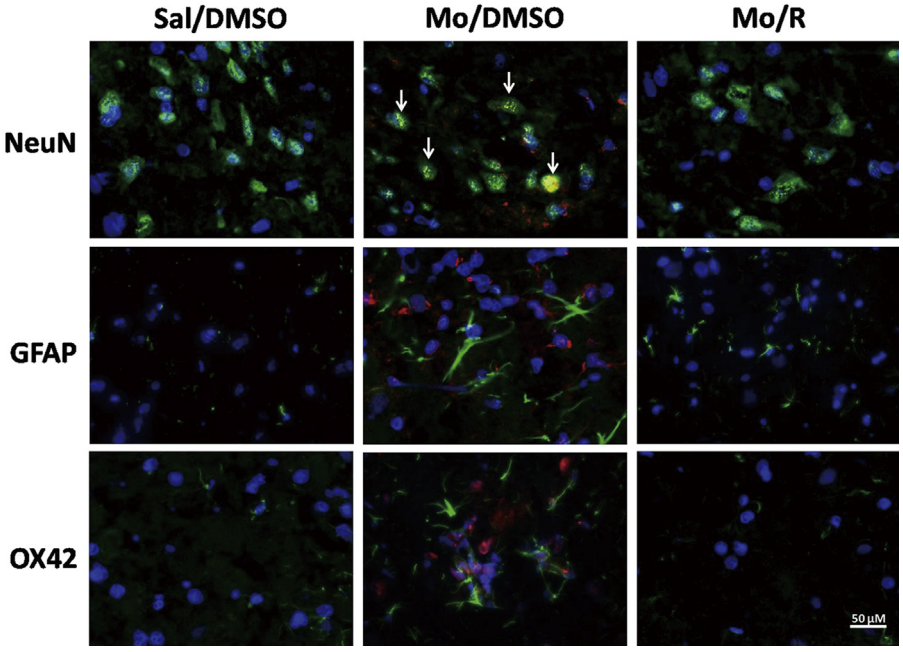

Figure 9 Resveratrol reverses morphine infusion-induced neuroinflammation. The infusion/injection protocol described in the legend to Figure 5 was used, then samples were collected 120 minutes after morphine challenge and sections of the dorsal horn double-labeled for TNFR1 (red labeling) and NeuN (neurons, top row), GFAP (astrocytes, middle row), or OX42 (microglia, bottom row) (green labeling); overlap of the two labels is seen as yellow (white arrows). Left panels, control group (Sal/DMSO); central panels, morphine-tolerant group (Mo/DMSO); right panels, morphine infusion plus resveratrol injection (Mo/R). The results are representative of those for samples from three rats. Scale bar = 50 μm. DMSO = dimethyl sulfoxide; TNFR1 = tumor necrosis factor receptor 1.

expression following the development of morphine tolerance has not. In the present study, we demonstrated that an increase in mRNA and protein levels of TNF-α and TNFR1, but not TNFR2, was seen in the spinal cord after 24 hours of morphine infusion and persisted throughout the 5 days of infusion. This suggests that TNFR1, but not TNFR2, is involved in TNF-α-mediated signaling transduction in morphine tolerance induction and maintenance. Silva et al[36] found that resveratrol acts as a natural anti-TNF-α molecule and inhibits proinflammatory cytokine expression in TNF-α-treated dendritic cells by inhibiting NF-κB translocation to the nucleus. Our results also showed that resveratrol treatment of morphine-tolerant rats converted activated spinal glia to an inactive ramified state. Moreover, resveratrol reversed the morphine-induced increase in TNFR1 expression seen in neurons, but not in glia cells (both astrocytes and microglia). These results suggest that resveratrol can modify neuron—glia communication in morphine-induced neuroinflammation.

Histone deacetylation by HDACs, a common epigenetic modification of chromatin, is associated with expression of genes coding for cytokines.[37] The mammalian genome contains at least 18 HDAC genes, and the encoded proteins are differentially distributed in the nervous system.[38] HDAC inhibitors, which prevent the removal of acetyl groups from histones, result in amelioration of symptoms in many inflammatory diseases in animals.[39,40] These results suggest that regulation of histone acetylation might be useful in the management of clinical hyperalgesia. The results of our recent study[41] also support the idea that i.t. injection of baicalin, another Chinese herb with similar anti-inflammatory activity to resveratrol, results in significant reversal of the spinal nerve ligation-induced increase in HDAC1 expression, which is associated with restoration of the antinociceptive effect of morphine. Resveratrol has been reported to act as an HDAC1 inhibitor and to inhibit the inflammatory cascade and the expression of NF-κB, survivin, and sirtuin 1.[42—44] Similarly, our present study also showed that resveratrol reversed the morphine infusion-induced increases in HDAC1, TNF-α, and TNFR1 expression and glial cell activation, thus restoring the antinociceptive effect of morphine. Our results therefore suggest that resveratrol may regulate epigenetic processing, an effect that might be useful in clinical pain management.

This study is an integral first step in assessing the usefulness of resveratrol in morphine-evoked hyperalgesia and the association between TNF-α-related signaling and HDAC1 expression in morphine tolerance. It provides new evidence that resveratrol may have potential as an analgesic adjuvant in clinical pain management, particularly in patients who need long-term morphine treatment and in morphine-tolerant patients who require better pain relief.

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References

1. Wong CS. The mechanisms of ultra-low dose opioid agonist—antagonist. *J Formos Med Assoc* 2011;**110**:666. author reply 7.
2. Shen CH, Tsai RY, Tai YH, Lin SL, Chien CC, Wong CS. Intrathecal etanercept partially restores morphine's antinociception in morphine-tolerant rats via attenuation of the glutamatergic transmission. *Anesth Analg* 2011;**113**:184—90.
3. Wen YR, Tan PH, Cheng JK, Liu YC, Ji RR. Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance. *J Formos Med Assoc* 2011;**110**:487—94.
4. Tsai RY, Chou KY, Shen CH, Chien CC, Tsai WY, Huang YN, et al. Resveratrol regulates *N*-methyl-D-aspartate receptor expression and suppresses neuroinflammation in morphine-tolerant rats. *Anesth Analg* 2012;**115**:944—52.
5. Chou KY, Tsai RY, Tsai WY, Wu CT, Yeh CC, Cherng CH, et al. Ultra-low dose (+)-naloxone restores the thermal threshold of morphine tolerant rats. *J Formos Med Assoc* 2013;**112**:795—800.
6. Griffin RS, Costigan M, Brenner GJ, Ma CH, Scholz J, Moss A, et al. Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. *J Neurosci* 2007;**27**:8699—708.
7. Tsai RY, Shen CH, Feng YP, Chien CC, Lee SO, Tsai WY, et al. Ultra-low-dose naloxone enhances the antinociceptive effect of morphine in PTX-treated rats: regulation on global histone methylation. *Acta Anaesthesiol Taiwan* 2012;**50**:106—11.
8. Liang DY, Li X, Clark JD. Epigenetic regulation of opioid-induced hyperalgesia, dependence, and tolerance in mice. *J Pain* 2013;**14**:36—47.
9. Doehring A, Geisslinger G, Lotsch J. Epigenetics in pain and analgesia: an imminent research field. *Eur J Pain* 2011;**15**:11—6.
10. Chen YW, Hsieh PL, Chen YC, Hung CH, Cheng JT. Physical exercise induces excess hsp72 expression and delays the development of hyperalgesia and allodynia in painful diabetic neuropathy rats. *Anesth Analg* 2013;**116**:482—90.
11. Leoni F, Fossati G, Lewis EC, Lee JK, Porro G, Pagani P, et al. The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo. *Mol Med* 2005;**11**:1—15.
12. Jiang LY, Li SR, Zhao FY, Spanswick D, Lin MT. Norepinephrine can act via alpha(2)-adrenoceptors to reduce the hyperexcitability of spinal dorsal horn neurons following chronic nerve injury. *J Formos Med Assoc* 2010;**109**:438—45.
13. Sato KL, Johanek LM, Sanada LS, Sluka KA. Spinal cord stimulation reduces mechanical hyperalgesia and glial cell activation in animals with neuropathic pain. *Anesth Analg* 2014;**118**:464—72.
14. Haydon PG, Blendy J, Moss SJ, Rob Jackson F. Astrocytic control of synaptic transmission and plasticity: a target for drugs of abuse? *Neuropharmacology* 2009;**56**:83—90.
15. Shen CH, Tsai RY, Shih MS, Lin SL, Tai YH, Chien CC, et al. Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphine-tolerant rats. *Anesth Analg* 2011;**112**:454—9.
16. Perez-Severiano F, Bermudez-Ocana DY, Lopez-Sanchez P, Rios C, Granados-Soto V. Spinal nerve ligation reduces nitric oxide synthase activity and expression: effect of resveratrol. *Pharmacol Biochem Behav* 2008;**90**:742—7.
17. Pervaiz S. Resveratrol: from grapevines to mammalian biology. *FASEB J* 2003;**17**:1975—85.
18. Kumar A, Kaundal RK, Iyer S, Sharma SS. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. *Life Sci* 2007;**80**:1236—44.
19. Grossman ML, Basbaum AI, Fields HL. Afferent and efferent connections of the rat tail flick reflex (a model used to

analyze pain control mechanisms). *J Comp Neurol* 1982;**206**:9—16.
20. Tsai RY, Jang FL, Tai YH, Lin SL, Shen CH, Wong CS. Ultra-low-dose naloxone restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in PTX-treated rats. *Neuropsychopharmacology* 2008;**33**:2772—82.
21. Tsai RY, Tai YH, Tzeng JI, Cherng CH, Yeh CC, Wong CS. Ultra-low dose naloxone restores the antinociceptive effect of morphine in pertussis toxin-treated rats by reversing the coupling of mu-opioid receptors from Gs-protein to coupling to Gi-protein. *Neuroscience* 2009;**164**:435—43.
22. Bermudez-Ocana DY, Ambriz-Tututi M, Perez-Severiano F, Granados-Soto V. Pharmacological evidence for the participation of NO-cyclic GMP-PKG-K^+ channel pathway in the anti-allodynic action of resveratrol. *Pharmacol Biochem Behav* 2006;**84**:535—42.
23. Pham-Marcou TA, Beloeil H, Sun X, Gentili M, Yaici D, Benoit G, et al. Antinociceptive effect of resveratrol in carrageenan-evoked hyperalgesia in rats: prolonged effect related to COX-2 expression impairment. *Pain* 2008;**140**:274—83.
24. Raghavendra V, Tanga FY, DeLeo JA. Attenuation of morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammatory immune responses by propentofylline in rats. *Neuropsychopharmacology* 2004;**29**:327—34.
25. Johnston IN, Milligan ED, Wieseler-Frank J, Frank MG, Zapata V, Campisi J, et al. A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. *J Neurosci* 2004;**24**:7353—65.
26. Wang Z, Ma W, Chabot JG, Quirion R. Cell-type specific activation of p38 and ERK mediates calcitonin gene-related peptide involvement in tolerance to morphine-induced analgesia. *FASEB J* 2009;**23**:2576—86.
27. Tai YH, Tsai RY, Lin SL, Yeh CC, Wang JJ, Tao PL, et al. Amitriptyline suppressesses neuroinflammation-dependent interleukin-10-p38 mitogen-activated protein kinase-heme oxygenase-1 signaling pathway in chronic morphine infused rats. *Anesthesiology* 2009;**110**:1—11.
28. Lin TC, Lai SL, Hsu SP, Ro LS. Treatment of neuropathic pain in acute intermittent porphyria with gabapentin. *J Formos Med Assoc* 2013;**112**:578—9.
29. Song P, Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res* 2001;**39**:281—6.
30. Tai YH, Cheng PY, Tsai RY, Chen YF, Wong CS. Purinergic P2X receptor regulates *N*-methyl-D-aspartate receptor expression and synaptic excitatory amino acid concentration in morphine-tolerant rats. *Anesthesiology* 2011;**113**:1163—75.
31. Tai YH, Wang YH, Tsai RY, Wang JJ, Tao PL, Liu TM, et al. Amitriptyline preserves morphine's antinociceptive effect by regulating the glutamate transporter GLAST and GLT-1 trafficking and excitatory amino acids concentration in morphine-tolerant rats. *Pain* 2007;**129**:343—54.
32. Tai YH, Tsai RY, Wang YH, Cherng CH, Tao PL, Liu TM, et al. Amitriptyline induces nuclear transcription factor-kappaB-dependent glutamate transporter upregulation in chronic morphine-infused rats. *Neuroscience* 2008;**153**:823—31.
33. Figiel I, Dzwonek K. TNFalpha and TNF receptor 1 expression in the mixed neuronal—glial cultures of hippocampal dentate gyrus exposed to glutamate or trimethyltin. *Brain Res* 2007;**1131**:17—28.
34. Sommer C, Schmidt C, George A. Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1. *Exp Neurol* 1998;**151**:138—42.
35. Yang CP, Cherng CH, Wu CT, Huang HY, Tao PL, Lee SO, et al. Intrathecal ultra-low dose naloxone enhances the anti-hyperalgesic effects of morphine and attenuates tumor necrosis factor-alpha and tumor necrosis factor-alpha receptor 1 expression in the dorsal horn of rats with partial sciatic nerve transection. *Anesth Analg* 2013;**117**:1493—502.
36. Silva AM, Oliveira MI, Sette L, Almeida CR, Oliveira MJ, Barbosa MA, et al. Resveratrol as a natural anti-tumor necrosis factor-alpha molecule: implications to dendritic cells and their crosstalk with mesenchymal stromal cells. *PloS One* 2014;**9**:e91406.
37. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet* 2009;**10**:32—42.
38. Renthal W, Maze I, Krishnan V, Covington 3rd HE, Xiao G, Kumar A, et al. Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron* 2007;**56**:517—29.
39. Chung YL, Lee MY, Wang AJ, Yao LF. A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. *Mol Ther* 2003;**8**:707—17.
40. Glauben R, Batra A, Fedke I, Zeitz M, Lehr HA, Leoni F, et al. Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *J Immunol* 2006;**176**:5015—22.
41. Cherng CH, Lee KC, Chien CC, Chou KY, Cheng YC, Hsin ST, et al. Baicalin ameliorates neuropathic pain by suppressing HDAC1 expression in the spinal cord of spinal nerve ligation rats. *J Formos Med Assoc* 2014;**113**:513—20.
42. Fulda S. Resveratrol and derivatives for the prevention and treatment of cancer. *Drug Discov Ttoday* 2010;**15**:757—65.
43. Venturelli S, Berger A, Bocker A, Busch C, Weiland T, Noor S, et al. Resveratrol as a pan-HDAC inhibitor alters the acetylation status of jistone proteins in human-derived hepatoblastoma cells. *PloS One* 2013;**8**:e73097.
44. Youn J, Lee JS, Na HK, Kundu JK, Surh YJ. Resveratrol and piceatannol inhibit iNOS expression and NF-kappaB activation in dextran sulfate sodium-induced mouse colitis. *Nutr Cancer* 2009;**61**:847—54.