Contribution of acid sphingomyelinase in the periaqueductal gray region to morphine-induced analgesia in mice

Joseph K. Ritter, Youwen Fang, Min Xia, Pin-Lan Li and William L. Dewey

Opioids are the most widely used drugs for long-term pain management, but their use is limited by the development of antinociceptive tolerance. The present study investigated the role of ceramide production through acid sphingomyelinase (ASM) activation in the periaqueductal gray region, a brain region implicated in opioid analgesia and tolerance. Morphine treatment was found, using immunohistochemistry, to increase ASM expression and intracellular ceramide in the periaqueductal gray 30 min after an acute injection (10 mg/kg). The effects of acute morphine treatment on ASM expression and ceramide generation in the periaqueductal gray region were completely blocked by pretreatment with naloxone and by silencing the ASM gene by plasmid-mediated transfection of ASM shRNA. In chronic morphine pellet-implanted mice, ASM expression and ceramide generation in the periaqueductal gray region were also significantly increased. Functionally, selective silencing of the ASM gene by local ASM shRNA transfection reduced the analgesic response to acute morphine, but the data on the effect of ASM shRNA on the development of antinociceptive tolerance were inconclusive. These data provide evidence that ASM activation and ceramide generation in the periaqueductal gray region play a major role in the antinociceptive mechanism of morphine. *NeuroReport* **23:780–785 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Opioids are widely relied upon for relief of moderate to severe pain. However, their long-term utility for chronic therapy is limited by the development of tolerance, which necessitates the use of increasing doses to achieve the same level of pain relief. The mechanisms underlying the antinociceptive effect of opioids and development of tolerance are complex and not yet completely understood. The opioids exert their actions at multiple levels of the central nervous system, including effects on both ascending and descending pain transmission pathways. These include the periaqueductal gray region, ventral lateral nucleus, and superficial laminae of the dorsal horn of the spinal cord [1]. The importance of the periaqueductal gray region in analgesic mechanisms of opioids [2] is emphasized by its high density of μ-opioid receptors and the observation that microinjection of opioids into this region exerts profound antinociceptive effects, which are prevented by naloxone, a μ-opioid receptor antagonist [3].

At the cellular level, μ-opioid receptors are present in lipid rafts, discrete micromembrane domains characterized by a high content of sphingolipids and cholesterol [4,5]. Lipid rafts are proposed to maximize the efficiency of receptor signaling, because the rafts concentrate receptors together with their coupling and effector proteins. As many receptors in lipid rafts respond to stimulation by triggering the activation of acid sphingomyelinase (ASM) and the generation of ceramide [6], we hypothesized that morphine stimulation of μ-opioid receptors could lead to the activation of ASM and ceramide generation and this could contribute toward the mechanism of morphine-induced antinociception and development of antinociceptive tolerance. Indeed, repeated administration of morphine was shown to increase the levels of ceramide in the dorsal horn of the spinal cord, an effect attributable in part to the activation of ASM [7]. In the present study, the effect of morphine on ASM expression and ceramide generation in the periaqueductal gray region was characterized by immunohistochemistry, and the role of ASM and ceramide generation in the mechanism of morphine antinociception and development of tolerance was assessed.

Materials and methods

Drugs and chemicals

The placebo and 75 mg morphine pellets were obtained from the National Institute on Drug Abuse (Bethesda, Maryland, USA). Morphine sulfate (Mallinckrodt, St Louis, Missouri, USA) was dissolved in pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, Illinois, USA).

Animals and drug treatments

Male Swiss-Webster mice (Harlan, Indianapolis, Indiana, USA), weighing 25–30 g, were maintained at $22 \pm 2^{\circ}$C on a 12-h light/dark cycle. Food and water were provided

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ad libitum. All procedures involving mice were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and complied with the recommendations of the International Association for the Study of Pain (Seattle, Washington, USA). Mice received subcutaneous injections of either saline (vehicle control) or morphine (10 mg/kg), and 30 min later, were tested in the tail flick assay or euthanized for immunohistochemical analysis of ASM and ceramide in brain tissue. The effect of naloxone was tested by administering 1 mg/kg subcutaneously for 30 min before the vehicle or the morphine injection. Antinociceptive tolerance to morphine was induced by surgical subcutaneous implantation of either placebo or morphine pellets. After 7 days, the mice were euthanized for immunohistochemical analysis of brain tissue or were injected with acute saline or morphine (10 mg/kg, subcutaneously), and 30 min later, tested in the tail flick assay.

Immunohistochemistry

The effect of morphine on ASM expression and ceramide generation was studied using standard immunohistochemical methods. Briefly, sections (5 μm) of brain from paraffin-embedded tissues were prepared and stained using antibodies against ceramide [1 : 50(v/v) in PBS (Sigma-Aldrich, St Louis, Missouri, USA)] or ASM [1 : 50(v/v) in PBS (Santa Cruz Biotech Inc., Santa Cruz, California, USA)]. Positive staining was detected as a brown color formed from the reaction of diaminobenzidine with horseradish peroxidase. To verify the binding specificity for ceramide and ASM, some sections were also incubated with only the primary antibody or with only the secondary antibody. In these cases, no positive staining was found in the sections, indicating that the immunoreactions were positive in all experiments carried out.

Warm water tail flick assay

The antinociceptive response to morphine was determined using the warm water tail-immersion test performed as described using a temperature-controlled water bath (56 ± 0.1°C) [8]. Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time from 2 to 4 s were used. The average baseline latency for these experiments was 3.0 ± 0.1 s. The test latency after drug treatment was assessed 30 min after the 10 mg/kg morphine challenge dose, with a 10-s maximal cut-off time to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson as the percentage of the maximal possible effect (%MPE), which was calculated as [(test latency − control latency)/(10 − control latency)] × 100 [9].

shRNA-mediated silencing of the ASM gene

The ASM shRNA used in this study was purchased from Sigma-Aldrich and was proved to knock down ASM gene expression in mammalian cells by Sigma-Aldrich and in mouse epithelial cells by our lab (data not shown). The sequence of the ASM shRNA was 5′-CCG GCC TTC ATA AAG GGA AAC CAA TCT CGA GAT TGG TTT CCC TTT ATG AAG GTT TTT G-3′. For a control, a scrambled small RNA with the sequence 5′-AAT TCT CCG AAC GTG TCA CGT-3′ was used and also confirmed as a nonsilencing double-stranded RNA.

Mice were transfected by an injection of shRNA or control RNA into the lateral ventricle as described [10]. Briefly, after anesthesia with ketamine/xylazine, a horizontal incision was made across the scalp, and a free-hand 5-μl injection of ASM shRNA or scrambled RNA plus + in-vivo jetPEI transfection reagent was administered 2 mm rostral and 2 mm lateral at a 45° angle from bregma. Our laboratory routinely uses the intracerebroventricular route of administration with high accuracy [11–14]. Five days later, the mice were subjected to acute or chronic morphine exposure experiments as described above. To confirm the effectiveness of the transfection method, a luciferase reporter plasmid was used. After 5 days, the mice were anesthetized and injected with luciferin (150 mg/kg, intraperitoneally), followed by in-vivo imaging using an IVIS200 imaging system (Xenogen Corp., Alameda, California, USA). Strong light emission was observed in a localized region of the brain, which was confirmed by the dissection of periaqueductal gray tissue and reanalysis using the IVIS system (data not shown).

Statistical analysis

Data are reported as the mean ± SEM. Significant differences between and within multiple groups were examined using analysis of variance for repeated measurements, followed by Duncan's multiple-range test. Student's *t*-test or the Mann–Whitney rank sum test was used to evaluate for a significant difference between two groups (for normally and non-normally distributed sample populations, respectively). For *P* value of less than 0.05 was considered statistically significant.

Results

Acute morphine treatment increased ASM expression and ceramide generation in the periaqueductal gray region

As repeated morphine administration was associated previously with increased ceramide levels in the dorsal horn of the spinal cord [7], the effect of morphine on ASM expression and ceramide generation in the periaqueductal gray region and the cortex was evaluated by immunohistochemistry. Figure 1 presents the representative images showing the effect of acute morphine treatment. Both ASM and ceramide were markedly elevated in the periaqueductal gray but not the cortex with morphine treatment. Quantification of the staining indicated a remarkably close agreement in the extents of ASM and ceramide elevation by morphine (42 and 39%, respectively).

These effects were abolished in animals pretreated with naloxone.

Silencing of ASM gene blocked the effects of acute morphine treatment on ASM expression and ceramide generation

The effect of ASM gene silencing on the responses of local ASM expression and ceramide generation to morphine was tested by transfecting either the control RNA or ASM shRNA and allowing the animals to recover for 5 days before an acute morphine dose. The transfection efficiency of shRNA was monitored by in-vivo molecular imaging and then confirmed by real-time PCR and Western blot analysis of local tissues at the end of each experiment. Morphine treatment of the mice transfected with the control RNA significantly elevated ASM and ceramide staining in the periaqueductal gray but not the cortex. Quantified data showing this effect are presented in Fig. 2. The percentage increase in ASM and ceramide again was observed to be very similar (48 vs. 54%, respectively), supporting a close relationship. In contrast, no effect of morphine on ASM and ceramide staining was evident in the ASM-transfected mice.

ASM gene silencing inhibited the antinociceptive response to acute morphine

In untransfected control mice (Fig. 3a), a single acute administration of morphine increased the %MPE from 3 to 98%, and this effect was blocked by naloxone pretreatment. In mice transfected with the scrambled control RNA (Fig. 3b), morphine increased the tail flick latency. The tail flick response was blunted in mice transfected with the ASM shRNA construct.

Chronic morphine exposure elevated acid sphingomyelinase expression and ceramide generation in the periaqueductal gray region

The effect of a 7-day exposure to a placebo or morphine pellet on ASM expression and ceramide generation was evaluated (Fig. 4). The chronic morphine-exposed mice showed elevations in ASM (Fig. 4a) and ceramide staining (Fig. 4b) in the periaqueductal gray region that were comparable in magnitude. In contrast, there was no significant effect of chronic morphine exposure on ASM or ceramide staining in the cortex.

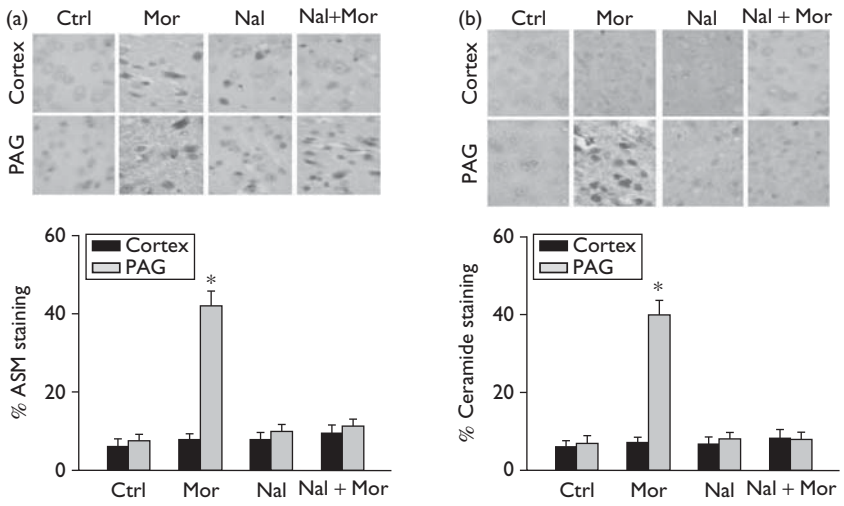
Effect of ASM gene silencing on the responses to morphine challenge doses in placebo and morphine pellet-exposed mice

Mice transfected with the scrambled control RNA and exposed for 7 days to an implanted morphine pellet showed a 67% decrease in %MPE to the morphine challenge dose compared with the corresponding placebo pellet-exposed controls (Fig. 5). In contrast, the %MPE in mice transfected with ASM shRNA and exposed for 7 days to the morphine pellet did not differ significantly from the corresponding ASM shRNA-transfected, placebo pellet-exposed mice.

Discussion

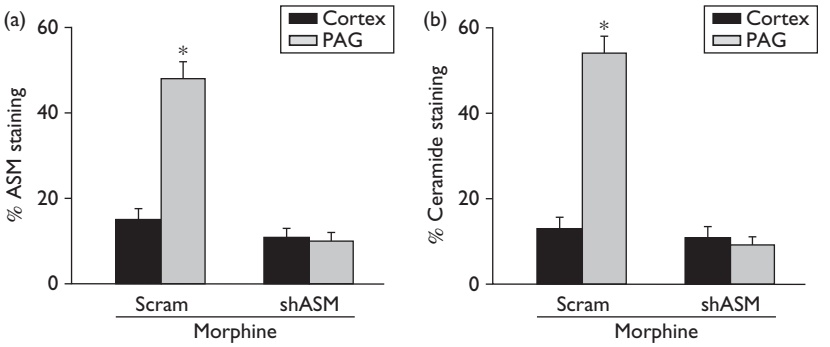
This work shows that morphine treatment increased the levels of ASM and ceramide in the periaqueductal gray,

Fig. 1



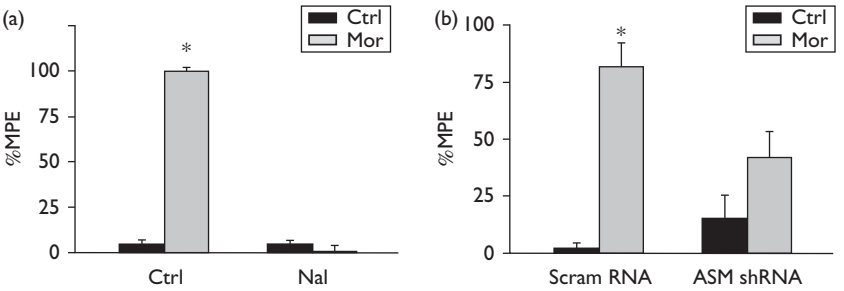
Acid sphingomyelinase (ASM) and ceramide are increased in the periaqueductal gray (PAG) region after acute morphine treatment. Mice received vehicle (Ctrl) or morphine (Mor) and, 30 min later, were euthanized for immunohistochemical analysis of ASM (a) or ceramide (b). Groups of mice received naloxone (Nal) 30 min before the vehicle or Mor. Top, representative images. Bottom, summarized data representing the mean±SEM of $n=5$ mice per group expressed as % ASM or ceramide positive-stained cells. *$P<0.05$ versus respective Ctrl group, analysis of variance ($F=48.207$ and 47.075 for ASM and ceramide, respectively, with 3 *d.f.*).

Fig. 2



Silencing of acid sphingomyelinase (ASM) gene expression blocks the effects of acute morphine treatment on ASM and ceramide in the periaqueductal gray (PAG) region. Mice were transfected with either scrambled (Scram) control RNA or ASM shRNA. After 5 days, the mice were treated with morphine and, 30 min later, euthanized for immunohistochemical analysis of ASM (a) or ceramide (b) in the PAG region or the cortex. Summarized data are presented as the mean±SEM of $n=6$ mice per group. *$P<0.001$ versus respective ASM shRNA group, *t*-test.

Fig. 3

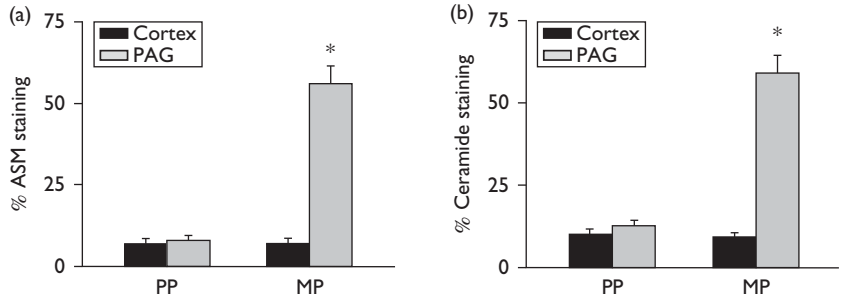


Naloxone (Nal) and acid sphingomyelinase (ASM) gene silencing reduce the antinociceptive response of morphine (Mor) in the tail flick assay. Mice were treated with Mor, and 30 min later, the antinociceptive effect was measured using the tail flick assay. (a) Effect of pretreatment with saline (Ctrl) or Nal 30 min before Mor. (b) Effect of local transfection with scrambled (Scram) control RNA or ASM shRNA. Each bar represents the mean±SEM expressed as the percentage of the maximum possible effect (%MPE), $n=5$ per group. *$P<0.05$ versus respective Ctrl group, rank sum test.

a brain region implicated in the supraspinal mechanisms of opioid-induced antinociception [15]. These effects were blocked by naloxone, suggesting that it is mediated by the activation of μ-opioid receptors. The selectivity of the effect was evident in that it occurred in the periaqueductal region but not in a brain region with lower μ-opioid receptor expression (cortex). The finding that shRNA-mediated silencing of the ASM gene prevented the increases in both ASM expression and ceramide generation in response to morphine is consistent with the hypothesis that the elevated ceramide is because of increased enzymatic hydrolysis of sphingomyelin. The effect of opioid treatment on ASM expression and ceramide generation occurred simultaneously with the production of antinociception by 30 min after the administration of morphine, and also after the chronic exposure of mice to morphine through an implanted morphine pellet. These data suggest that little tolerance to the effects on ASM expression and ceramide generation develops as a result of chronic opioid exposure, which contrasts to the marked antinociceptive tolerance that develops after chronic morphine.

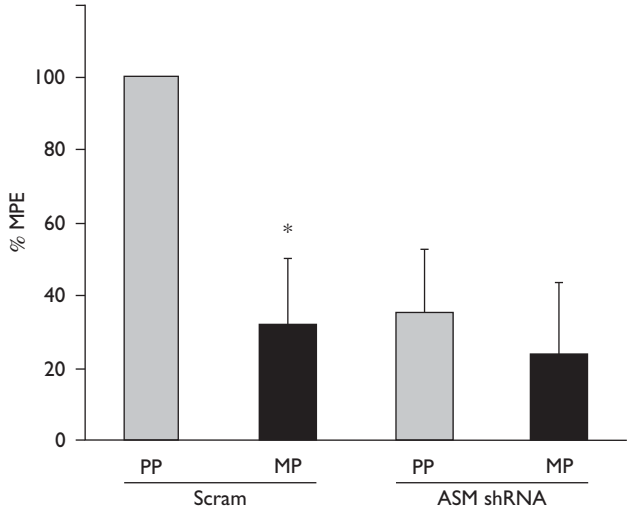
The present study shows a role for ASM activation and ceramide generation in the antinociceptive mechanism of morphine in that the localized transfection of ASM shRNA, which prevented the morphine-induced elevations in both ASM and ceramide of the periaqueductal gray, reduced the %MPE response to the morphine challenge dose as measured by the tail flick assay. The data are inconclusive, however, on whether inhibition of the ASM/ceramide pathway can impact the development of tolerance to morphine. Although mice transfected with the ASM shRNA and exposed for 7 days to the morphine pellet showed a %MPE that did not differ from that of the ASM-transfected, placebo pellet-exposed mice, the interpretation was confounded by the reduction in antinociceptive activity observed in the ASM shRNA-transfected, placebo pellet-exposed mice. In previous studies, ceramide was shown to play a role in the

Fig. 4



Acid sphingomyelinase (ASM) and ceramide are increased in the periaqueductal gray (PAG) region after chronic exposure to morphine. Mice were implanted with a placebo pellet (PP) or a morphine pellet (MP) and, 7 days later, were euthanized for analysis of (a) ASM or (b) ceramide by immunohistochemistry. The bars represent the mean±SEM, $n=6$ per group. *$P<0.001$ versus respective PP group, *t*-test.

Fig. 5



Effect of acid sphingomyelinase (ASM) gene silencing on antinociceptive responses in acute and chronic morphine-exposed mice in the tail flick assay. Mice were transfected with either scrambled (Scram) control RNA or ASM shRNA, and 5 days later, the mice were implanted with placebo or morphine pellets (MP). After 7 days, the mice were treated with acute morphine and tested 30 min later in the tail flick assay. The bars represent the mean±SEM, $n=5$ per group. %MPE, percentage of the maximum possible effect. *$P<0.05$ versus respective placebo pellet (PP) group, rank sum test.

mechanism of tolerance development to morphine in the dorsal horn of the spinal cord [7]. Repeated administration of morphine to mice increased ceramide levels in dorsal horn tissue, and inhibition of ASM activity with a pharmacologic inhibitor blocked the effect and appeared to reverse tolerance. Ceramide generated in response to repeated morphine is proposed to contribute toward tolerance development through a mechanism involving the induction of cytokines, neuroimmune/neuroglial activation, and apoptosis of cells in the spinal cord [16,17]. The findings in the present study complement previous reports by showing that morphine treatment activates ASM to generate ceramide in different tissues implicated in the mechanism of opioids. However, increased ASM expression and ceramide generation in the periaqueductal gray region may be mainly responsible for the analgesic action of morphine, which is different from its action in the dorsal horn of the spinal cord that contributes toward the development of morphine tolerance.

Conclusion

In the present study, the administration of morphine was shown to increase the levels of ASM and ceramide in the periaqueductal gray region, which contributes toward the antinociceptive efficacy of morphine. The data suggest a role of membrane raft clustering and the formation of ceramide-enriched signaling platforms in the mechanism of μ-opioid receptor-induced antinociception.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

References

1 Yaksh TL. Pharmacology and mechanisms of opioid analgesic activity. *Acta Anaesthesiol Scand* 1997; **41**:94–111.
2 Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu Rev Neurosci* 1984; **7**:309–338.
3 Bodnar RJ, Williams CL, Lee SJ, Pasternak GW. Role of mu 1-opiate receptors in supraspinal opiate analgesia: a microinjection study. *Brain Res* 1988; **447**:25–34.
4 Zhao H, Loh HH, Law PY. Adenylyl cyclase superactivation induced by long-term treatment with opioid agonist is dependent on receptor localized within lipid rafts and is independent of receptor internalization. *Mol Pharmacol* 2006; **69**:1421–1432.
5 Qiu Y, Wang Y, Law PY, Chen HZ, Loh HH. Cholesterol regulates micro-opioid receptor-induced beta-arrestin 2 translocation to membrane lipid rafts. *Mol Pharmacol* 2011; **80**:210–218.

6 Gulbins E, Li PL. Physiological and pathophysiological aspects of ceramide. *Am J Physiol Regul Integr Comp Physiol* 2006; **290**:R11–R26.
7 Ndengele MM, Cuzzocrea S, Masini E, Vinci MC, Esposito E, Muscoli C, *et al*. Spinal ceramide modulates the development of morphine antinociceptive tolerance via peroxynitrite-mediated nitroxidative stress and neuroimmune activation. *J Pharmacol Exp Ther* 2009; **329**: 64–75.
8 Coderre TJ, Rollman GB. Naloxone hyperalgesia and stress-induced analgesia in rats. *Life Sci* 1983; **32**:2139–2146.
9 Harris LS, Pierson AK. Some narcotic antagonists in the benzomorphan series. *J Pharmacol Exp Ther* 1964; **143**:141–148.
10 Pedigo NW, Dewey WL, Harris LS. Determination and characterization of the antinociceptive activity of intraventricularly administered acetylcholine in mice. *J Pharmacol Exp Ther* 1975; **193**:845–852.
11 Smith FL, Lohmann AB, Dewey WL. Involvement of phospholipid signal transduction pathways in morphine tolerance in mice. *Br J Pharmacol* 1999; **128**:220–226.
12 Smith FL, Javed R, Elzey MJ, Welch SP, Selley D, Sim-Selley L, *et al*. Prolonged reversal of morphine tolerance with no reversal of dependence by protein kinase C inhibitors. *Brain Res* 2002; **958**:28–35.
13 Smith FL, Javed RR, Elzey MJ, Dewey WL. The expression of a high level of morphine antinociceptive tolerance in mice involves both PKC and PKA. *Brain Res* 2003; **985**:78–88.
14 Smith FL, Javed RR, Smith PA, Dewey WL, Gabra BH. PKC and PKA inhibitors reinstate morphine-induced behaviors in morphine tolerant mice. *Pharmacol Res* 2006; **54**:474–480.
15 Benarroch EE. *Basic neurosciences with clinical applications*. Philadelphia, PA: Elsevier Health Sciences; 2006.
16 Bryant L, Doyle T, Chen Z, Cuzzocrea S, Masini E, Vinci MC, *et al*. Spinal ceramide and neuronal apoptosis in morphine antinociceptive tolerance. *Neurosci Lett* 2009; **463**:49–53.
17 Muscoli C, Doyle T, Dagostino C, Bryant L, Chen Z, Watkins LR, *et al*. Counter-regulation of opioid analgesia by glial-derived bioactive sphingolipids. *J Neurosci* 2010; **30**:15400–15408.