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Effects of morphine on formalin-induced nociception in rats

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Abstract

This study focused on the antinociceptive action of morphine in the formalin test in rats. Formalin-induced behaviour is characterised by two phases relevant to acute and tonic pain. Morphine (1–6 mg/kg) was administered systemically before or after the early phase, and its ability to affect the late phase was investigated. Inhibitory effects of morphine (3 mg/kg) injected immediately after the early phase were significantly stronger (32 ± 9%) compared to the preemptive administration (84 ± 29%, relative to saline-treated controls, 5% formaldehyde). It appears that some neural and/or behavioural changes during the early phase limit effects of morphine on the late phase. Furthermore, manipulation of stimulation intensity (2% vs. 5% formaldehyde) significantly affected the ability of morphine (3 mg/kg) to suppress early (55 ± 7% and 76 ± 10%, respectively) but not late phase of formalin-induced behaviours. These results agree with the previous demonstrations on the effects of acute nociceptive stimulation intensity on analgesic potency of opiate drugs. Thus, the present study revealed two factors that affect the potency of morphine in formalin test: administration regimen and formalin concentration.

Keywords: Opiate; Morphine; Pain; Analgesia; Formalin test; (Rat)

1. Introduction

There is considerable clinical evidence that opiate analgesics differentially affect responses to pain produced by brief noxious stimulation and those associated with persistent pain states. Acute pain is usually highly sensitive to opiate therapy (Becker et al., 2000) yet high doses of opiates are required to relieve the nociceptive pain (Sosnowski, 1993) and some clinical and laboratory studies indicate that it is inherently resistant to opiate therapy (Becker et al., 2000). Nociceptive type of neuropathic pain can be caused by different mechanisms, including metastatic cancer, nerve compression or inflammation (Dellemijn, 1999), and can be observed as an increased response to noxious stimulation (hyperalgesia), increased response duration (persistent pain) or a pain response to non-noxious stimulus (allodynia).

Laboratory methods used for the analysis of persistent pain are based upon a variety of nerve damage techniques (Bennett and Xie, 1988) or peripheral inflammation (Dubuisson and Dennis, 1977). One of the commonly used procedures is based on the assessment of behaviour induced by subcutaneous administration of aqueous formaldehyde solution (formalin) into the animal's paw or vibrissal pad (Dubuisson and Dennis, 1977).

The responses to formalin-induced pain, such as licking and biting of the injected paw, are biphasic. The first (early) phase is caused predominantly by C-fibre activation due to the peripheral stimulation (Martindale et al., 2001; McCall et al., 1996), while the second (late) phase appears to be dependent on ongoing stimulation of nociceptors and/or via spinal cord hyperexcitability which is dependent on *N*-methyl-D-aspartate (NMDA) receptor activation (Coderre et al., 1990; Coderre and Yashpal, 1994). The formalin test is especially useful for the within-subject observation of two consecutive and causally related phases of pain response over a short period of time (typically about 1 h). Therefore, it allows analysis of drug actions relevant for acute and tonic pain during one test.

In the formalin test, one can manipulate the pain intensity, for example, by changing the formalin concentration, in order to observe the actions of opiates on the expression of both the early and late phases. In the present study, lower formalin concentration was initially used in order to analyse antinoci-

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ceptive effects of morphine in the more moderate pain conditions.

Opiates such as morphine reliably inhibit behavioural, electrophysiological and biochemical responses evoked by acute thermal, mechanical and other noxious stimuli in naive animals (Ma et al., 1998; McCormack et al., 1998; Haley et al., 1990). Several studies demonstrated anti-nociceptive-like effects of morphine and related compounds on formalin-evoked behaviours. For instance, systemic morphine inhibited both the early and late phases of the formalin-induced licking response, and this activity was naloxone-sensitive (Dubuisson and Dennis, 1977; Oluyomi et al., 1992). Also, there are some publications which demonstrate that intrathecally applicated morphine was equieffective at inhibiting the second-phase formalin response in animals treated with morphine before formalin stimulation or immediately after the early phase (Chapman et al., 1994; Yamamoto and Yaksh, 1992). To the best of our knowledge, however, there were no studies comparing the effects of systemic opiates administered before or after the early phase in the formalin test.

The present study aimed to evaluate the effects of systemic morphine in the rat formalin test using different injection regimens and stimulation intensities.

2. Materials and methods

2.1. Subjects

Adult male drug- and experimentally naive Sprague–Dawley rats (170–250 g; Janvier, France) were housed in groups of four with food and water available ad libitum and alternating 12:12-h day–night cycle (lights on at 0700 h) for at least 3 days before the experiments were started. Colony room temperature and humidity were maintained at 20 ± 1 °C and $60 \pm 3\%$, respectively. All experiments were conducted during the light period of a day–night cycle. The study was approved by the Ethical Committee, Regirungspräesidium Darmstadt, Hessen, and were performed in accordance with the recommendations and policies of the U.S. National Institutes of Health Guidelines for the Use of Animals. Each animal was used only once.

2.2. Drugs

Formaldehyde, 5%, was made from 1 part formalin (~ 36.6%; formalin, Fluka) and 6.35 parts saline; formaldehyde, 2%, was made combining 1 part formalin and 17.3 of saline. Morphine sulphate (Sigma, Deisenhofen, Germany) was dissolved in physiological saline and injected subcutaneously (s.c.) in volume of 1 ml/kg. Microinjector syringes with 27-gauge needles were used for all injections.

2.3. Procedure

Rats were placed individually in an open Plexiglas chamber with a mirror angled at 45° positioned behind to allow an unobstructed view of the paws by the observer. The animals were habituated to the observation chamber for 30 min prior to the experimental sessions. Formalin (50 μl) was injected s.c. into the plantar surface of the rat hind paw (left or right, counterbalanced across each treatment group) using a 27-gauge needle. After injection, rats were immediately returned to the observation chamber and the formalin-induced behaviours were recorded by a trained observer continuously for 60 min. Formalin injection produced a characteristic behaviour consisting of flinching and licking/biting of the injected paw. In addition, the behaviour was videotaped during all periods of observation.

Separate groups of rats ($N = 7–10$ rats per group) received different doses of morphine or its vehicle prior to being injected with formalin. Morphine (1, 3 or 6 mg/kg) was administered 15, 30 or 60 min before or 6 min after the formalin injection. Formaldehyde concentration was either 2% or 5%.

2.4. Data analysis

Elementary analysis produced by the behavioural observation software yielded duration (in seconds) of recorded behaviours per each 6-min interval of the 60-min observation period. The 6-min interval was chosen based on earlier reports on the time-course of the early (0–6 min) and late (12–60 min) phases of the formalin-induced facial grooming (Eisenberg et al., 1996). Data were analysed using SAS-STAT software (ver. 6.11, SAS Institute, Cary, NC). Duration and frequencies of licking/biting the injected paw were subjected to the distribution-free analysis of variance (ANOVA) with repeated measures on observation intervals. For different experiments and corresponding analyses, independent variables included morphine dose, formalin concentration, and/or morphine injection times. Dunnett's and Tukey's tests were used for post hoc between-group comparisons whenever indicated by ANOVA.

3. Results

Injection of formalin into the hind paw of the rats induced biphasic behaviours of licking/biting and flinching/shaking observed between 0–6 min (early phase) and 12–60 min (late phase) with nearly no responses recorded between 6 and 12 min.

At the higher (5% formaldehyde) formalin concentration, morphine significantly affected the expression of both the early and late phases ($F(3,44) = 9.1$, $P < 0.01$; $F(3,44) = 8.9$, $P < 0.01$, respectively) (Fig. 1A). Post hoc tests confirmed that at the dose level of 6 mg/kg, morphine was capable of inhibiting both phases of formalin response (Fig. 1A).

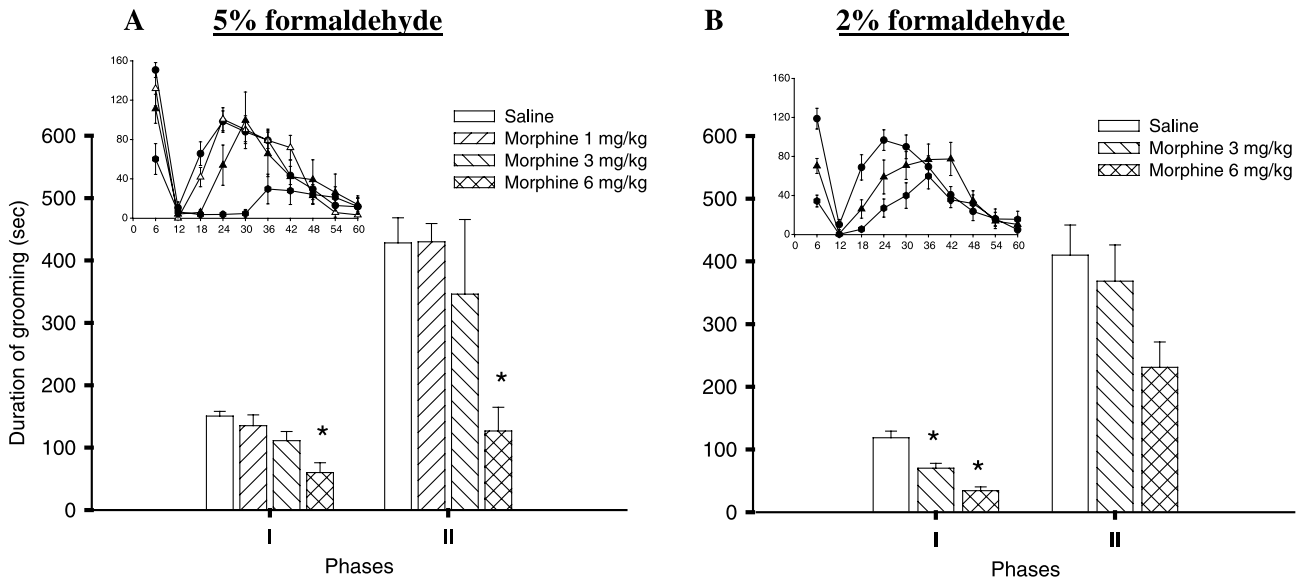

Fig. 1. Dose–effect relationships for the effects of morphine on formalin-induced behaviours. Morphine (1–6 mg/kg) or saline was given s.c. 30 min prior to the test. Behavioural observations started immediately after the formaldehyde injection of 2% (B) or 5% (A). Data are presented as mean ± S.E.M. durations (in seconds) of the injected paw licking and biting for the first and second phases (bar graphs) and for individual intervals (line graph inserts). For graph inserts: closed circles, saline; open triangles, morphine 1 mg/kg; closed triangles, morphine 3 mg/kg; closed diamonds, morphine 6 mg/kg (*$P<0.01$, compared to saline-treated group).

At the lower (2% formaldehyde) formalin concentration, morphine was less effective against the late phase of formalin response in contrast to the effects on the early phase responding (early phase, $F(2,48)=31.1$, $P<0.01$; late phase, $F(2,48)=3.2$, $P=0.052$). No dose of morphine affected the late phase responses, whereas both the 3 and 6 mg/kg doses suppressed the early phase (Fig. 1B). Visual inspection of the graphs suggested that effects of morphine on the late phase responding were limited to the first half of the session. ANOVA applied to the data collected during the first 18 min of the late phase (12–30 min) indicated that morphine dose-dependently inhibited the expression of this responding with both morphine doses achieving the level of statistical significance ($F(2,48)=11.6$, $P<0.01$).

To test the potential contribution of the pharmacokinetics or other time-dependent factors limiting the duration of morphine effects, various injection time intervals were used. Morphine (3 mg/kg) was administered 15, 30 or 60 min prior to formalin. As shown in Fig. 2, the effects of morphine did not depend on the presession in-

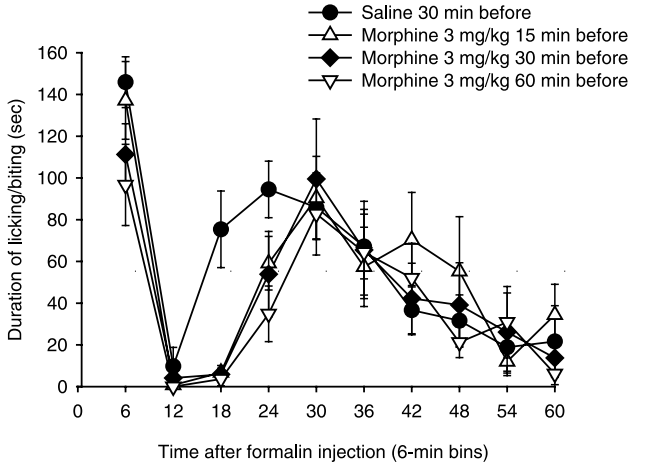

Fig. 2. Morphine-induced suppression of formalin behaviours: different presession injection times. Morphine (3 mg/kg) or saline was administered s.c. 15, 30 or 60 min prior to the test. Behavioural observations started immediately after the 5% formalin injection. Data are presented as mean ± S.E.M. durations (in seconds) of the injected paw licking and biting (see text for details).

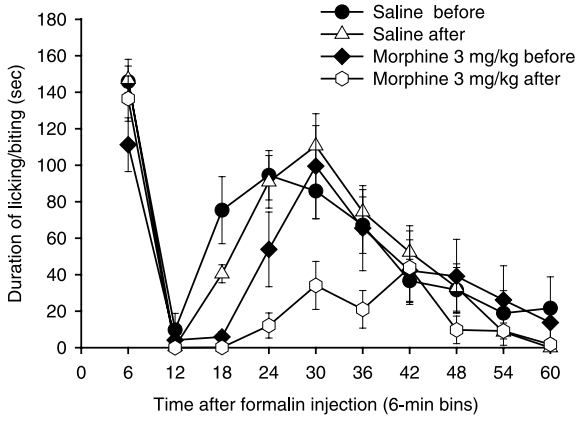

Fig. 3. Suppression of formalin-induced behaviours by morphine administered either before or after first phase of formalin response. Morphine (3 mg/kg) or saline was given s.c. either 30 min before or 6 min after the formalin injection. Behavioural observations started immediately after the formaldehyde (5%) injection. Data are presented as mean ± S.E.M. durations (in seconds) of the injected paw licking and biting (see text for details).

jection time (early phase, $F(2,21) = 1.4$; late phase, $F(2,21) = 0.2$).

The last set of experiments compared the effects of morphine administered before and after the first phase of formalin response (Fig. 3). The results demonstrated that the suppressive effects of morphine were more pronounced and lasted longer when the drug was injected immediately after the first phase (main effect of morphine: $F(3,31) = 9.1$, $P < 0.01$).

4. Discussion

There were two main findings revealed in the present study. First, the initial experiments showed relatively weak effects of pretreatment with 3 mg/kg morphine on the second phase of formalin test, which is believed to be relevant for tonic pain. This effect was clearly not dependent on pharmacokinetic factors or other time-dependent processes (acute tolerance-like) since it was independent from administration interval (15, 30, 60 min before the formalin injection). Further experiments brought that morphine was far more effective at suppressing the late phase of formalin-induced responding when it was administered after the early (acute) phase compared with the preemptive treatment. These results can probably be explained by neural and/or behavioural changes associated with the early phase, combined with the presence or an action of morphine, which somehow limits the effects of morphine on the late phase.

Over the last several years, a number of studies have pointed at activation of glutamatergic neurotransmission as a potential factor counteracting opiate analgesia. Although the present study employed no NMDA receptor ligands, it is likely that NMDA receptor activation is not the only but a principal contributing phenomenon, which can affect the expression of opiate analgesia. It has been repeatedly shown that antagonists acting at NMDA subtype of glutamate receptors enhance acute morphine analgesia (Price et al., 2000). Moreover, conditions known to up-regulate NMDA receptor function (e.g., persistent pain) are associated with reduced opiate analgesic efficacy in laboratory animals (Bennett, 2000). In the formalin test, glutamate released by the nociceptive afferents acts primarily on non-NMDA receptors and thereby produces conditions necessary for NMDA receptor activation via collateral stimulation (Millan, 1999). Alternatively, several reports suggest that opioid receptor stimulation via protein kinase-dependent mechanisms also facilitates NMDA receptor-mediated currents (Inoue and Ueda, 2000; Mayer et al., 1995; Vaccarino et al., 1991). NMDA receptor activation may then lead to altered coupling between opioid receptor and second messenger systems (Carroll et al., 1996; Malcangio and Bowery, 1999).

This prelude of NMDA receptor stimulation during the first phase is critical for the establishment and expression of the following late phase. A growing body of evidence shows that NMDA receptor antagonists prevent the development of persistent pain states and also selectively attenuate the expression of the late phase of formalin response (Davidson et al., 1997; Vaccarino and Couret, 1993; Vaccarino et al., 1993). Thus, in the case of morphine pretreatment, indirect facilitation by formalin of NMDA receptors coincides with opioid receptor stimulation by morphine and results in down-regulation of opioid receptor function and/or further up-regulation of NMDA receptors. This may explain the differences seen in the effects of morphine administered before and after the acute phase of formalin response.

Second important finding presented here concerns the effects of formalin concentration on responsiveness to morphine treatment. For instance, morphine seemed to have stronger effects against the early phase of responding induced by the lower formalin concentration (as opposed to the higher concentration of formalin). This can be possibly explained by morphine inhibiting the release of nociceptive transmitters via presynaptic μ-opioid receptors (Wheeler-Aceto and Cowan, 1991; Taylor et al., 2000). The early phase of formalin-induced behaviours is thought to be directly related to the C-fibre activation due to the peripheral stimulation (Martindale et al., 2001; McCall et al., 1996). It is conceivable that higher concentration of formalin produces a greater degree of stimulation and C-fibre activation. Accordingly, experimental data clearly indicated that lower concentration of formalin produced milder behavioural response compared to that induced by the higher concentration of formalin (118.75 ± 10.62 and 150.67 ± 7.56 s, respectively). Thus, morphine may be more effectively antagonizing the release of nociceptive transmitters induced by the lower degree of stimulation with the stronger stimulation overcoming these inhibitory influences. Indirect support for these speculations comes from the studies that noted similar dependence on the stimulation intensity for the effects of opiate analgesics in the tests of acute thermal nociception (Morgan et al., 1999; Nishiyama and Hanaoka, 2000).

In contrast, the second (late) phase of formalin-induced behaviours appears to be dependent on both functional changes in the dorsal horn of the spinal cord and peripheral inflammation (Coderre et al., 1990; Coderre and Yashpal, 1994). Thus, the late phase responding may be less dependent on, or less proportionate to, the magnitude of acute stimulation and C-fibre barrage. Indeed, our data suggest that the magnitude of the late-phase nociceptive behaviours was similar for groups injected with lower and higher formalin concentrations (410 ± 48 and 428 ± 40 s, respectively). Furthermore, morphine inhibited the late phase responding to the similar extent in rats treated with lower and higher formalin concentrations; this finding seems to be congruent with the explanations offered above for the differential effects of morphine against the early phase responding induced by lower and higher formalin concentrations.

Thus, the present study revealed two factors that affect the potency of morphine in the formalin test—administration regimen and formalin concentration. It appears that some neural activity during the early phase limits the effects of morphine on the formalin-induced behaviours. It can be speculated that the present results are relevant for the analysis of reduced opiate analgesia once the persistent pain states are established.

References

Becker, R., Jakob, D., Uhle, E.I., Riegel, T., Bertalanffy, H., 2000. The significance of intrathecal opioid therapy for the treatment of neuropathic cancer pain conditions. Stereotact. Funct. Neurosurg. 75, 16–26.

Bennett, G.J., 2000. Update on the neurophysiology of pain transmission and modulation: focus on the NMDA-receptor. J. Pain Symp. Manag. 19, S2–S6.

Bennett, G.J., Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

Carroll, F.Y., Beart, P.M., Cheung, N.S., 1996. NMDA-mediated activation of the NO/cGMP pathway: characteristics and regulation in cultured neocortical neurones. J. Neurosci. Res. 43, 623–631.

Chapman, V., Haley, J.E., Dickenson, A.H., 1994. Electrophysiologic analysis of preemptive effects of spinal opioids on *N*-methyl-D-aspartate receptor-mediated events. Anesthesiology 81, 1429–1435.

Coderre, T.J., Yashpal, K., 1994. Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model. Eur. J. Neurosci. 6, 1328–1334.

Coderre, T.J., Vaccarino, A.L., Melzack, R., 1990. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 535, 155–158.

Davidson, E.M., Coggeshall, R.E., Carlton, S.M., 1997. Peripheral NMDA and non-NMDA glutamate receptors contribute to nociceptive behaviors in the rat formalin test. NeuroReport 8, 941–946.

Dellemijn, P., 1999. Are opioids effective in relieving neuropathic pain? Pain 80, 453–462.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161–174.

Eisenberg, E., Vos, B.P., Strassman, A.M., 1996. The peripheral antinociceptive effect of morphine in a rat model of facial pain. Neuroscience 72, 519–525.

Haley, J.E., Sulliva, A.F., Dickenson, A.H., 1990. Evidence for spinal *N*-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. Brain Res. 518, 218–226.

Inoue, M., Ueda, H., 2000. Protein kinase C-mediated acute tolerance to peripheral mu-opioid analgesia in the bradykinin- test in mice. J. Pharmacol. Exp. Ther. 293, 662–669.

Ma, Q.P., Allchorne, A.J., Woolf, C.J., 1998. Morphine, the NMDA receptor antagonist MK801 and the tachykinin NK1 receptor antagonist RP67580 attenuate the development of inflammation-induced progressive tactile hypersensitivity. Pain 77, 49–57.

Malcangio, M., Bowery, N.G., 1999. Peptide autoreceptors: does an autoreceptor for substance P exist? Trends Pharmacol. Sci. 20, 405–407.

Martindale, J., Bland-Ward, P.A., Chessell, I.P., 2001. Inhibition of C-fibre mediated sensory transmission in the rat following intraplantar formalin. Neurosci. Lett. 316, 33–36.

Mayer, D.J., Mao, J.R., Price, D.D., 1995. The development of morphine tolerance and dependence is associated with translocation of protein kinase C. Pain 61, 365–374.

McCall, W.D., Tanner, K.D., Levine, J.D., 1996. Formalin induces biphasic activity in C-fibers in the rat. Neurosci. Lett. 208, 45–48.

McCormack, K., Prather, P., Chapleo, C., 1998. Some new insights into the effects of opioids in phasic and tonic nociceptive tests. Pain 78, 79–98.

Millan, M.J., 1999. The induction of pain: an integrative review. Prog. Neurobiol. 57, 1–164.

Morgan, D., Cook, C.D., Smith, M.A., Picker, M.J., 1999. An examination of the interactions between the antinociceptive effects of morphine and various mu-opioids: the role of intrinsic efficacy and stimulus intensity. Anesth. Analg. 88, 407–413.

Nishiyama, T., Hanaoka, K., 2000. The effects of epidural bupivacaine, morphine, and their combination on thermal nociception with different stimulus intensity in rats. Anesth. Analg. 91, 652–656.

Oluyomi, A.O., Hart, S.L., Smith, T.W., 1992. Differential antinociceptive effects of morphine and methylmorphine in the formalin test. Pain 49, 415–418.

Price, D.D., Mayer, D.J., Mao, J., Caruso, F.S., 2000. NMDA-receptor antagonists and opioid receptor interactions as related to analgesia and tolerance. J. Pain Symp. Manag. 19, S7–S11.

Sosnowski, M., 1993. Pain management: physiopathology, future research and endpoints. Support Care Cancer 1, 79–88.

Taylor, B.K., Peterson, M.A., Roderick, R.E., Tate, J., Green, P.G., Levine, J.O., Basbaum, A.I., 2000. Opioid inhibition of formalin-induced changes in plasma extravasation and local blood flow in rats. Pain 84, 263–270.

Vaccarino, A.L., Couret, L.C., 1993. Formalin-induced pain antagonizes the development of opiate dependence in the rat. Neurosci. Lett. 161, 195–198.

Vaccarino, F.M., Liljequist, S., Tallman, J.F., 1991. Modulation of protein kinase C translocation by excitatory and inhibitory amino acids in primary cultures of neurons. J. Neurochem. 57, 391–396.

Vaccarino, A.L., Marek, P., Kest, B., Weber, E., Keana, J.F.W., Liebeskind, J.C., 1993. NMDA receptor antagonists, MK-801 and ACEA-1011, prevent the development of tonic pain following subcutaneous formalin. Brain Res. 615, 331–334.

Wheeler-Aceto, H., Cowan, A., 1991. Neurogenic and tissue-mediated components of formalin-induced edema: evidence for supraspinal regulation. Agents Actions 34, 264–269.

Yamamoto, T., Yaksh, T.L., 1992. Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. Anesthesiology 77, 757–763.