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Electrophysiologic Analysis of Preemptive Effects of Spinal Opioids on N-methyl-D-aspartate Receptor–mediated Events

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Background: **Spinal N-methyl-D-aspartate (NMDA) receptor–mediated mechanisms may contribute to reduced opioid sensitivity in conditions of pain. The effectiveness of spinal opioids in inhibiting NMDA-mediated nociceptive events was assessed with two models. In addition, opioid dose–response curves with preemptive administration were compared with early and late postadministrations.**

Methods: **Dorsal horn nociceptive neuronal responses were recorded in the intact halothane anesthetized rat to acute repetitive C-fiber electrical stimulation (0.1 and 0.5 Hz) and to the peripheral injection of 5% formalin. At 0.5 Hz but not at 0.1 Hz, there was an enhanced C-fiber evoked response of dorsal horn neurons elicited by repetitive C-fiber stimulation (wind-up), which is mediated by the NMDA receptor. Formalin produced a biphasic response; the late protracted inflammatory phase was NMDA receptor–mediated.**

Results: **With 0.5-Hz stimulation a large degree of wind-up was elicited; it was less sensitive to 5 μg morphine compared with the effect of the same dose on the residual wind-up elicited at 0.1 Hz. Preadministration and early postadministration of morphine were equieffective at inhibiting the second-phase formalin response. In contrast, administration of the fast-acting μ opioid, D-Ala-Gly-MePHe-Gly-ol, given late postadministration (during the second phase) was less effective than preadministration. Increasing the dose of D-Ala-Gly-MePHe-Gly-ol produced complete inhibitions.**

Conclusions: **NMDA receptor–mediated neuronal responses, such as wind-up and the established second phase of the formalin response, are poorly responsive to opioids. Dose increases and preemptive opioids effectively inhibit these NMDA receptor–mediated events. (Key words: Analgesics: opioids. Pain: preemptive analgesia. Receptors: N-methyl-D-aspartate. Spinal cord.)**

THE effects of morphine are due to actions at μ-opioid receptors at peripheral, spinal and supraspinal sites.[1] The spinal analgesic action of morphine has been shown by numerous behavioral studies.[2] Microelectrophorectic administration of morphine at the level of the substantia gelatinosa of the dorsal horn selectively inhibits noxiously evoked responses of deep dorsal horn neurons.[3] *In vivo* electrophysiologic studies have shown the inhibitory actions of spinally acting μ-opioid agonists to be selective for C-fiber evoked responses of the dorsal horn neurons.[3–7]

Intrathecal opioids effectively inhibit the steady C-fiber evoked input responses of the dorsal horn neurons.[5] In contrast, enhanced C-fiber evoked responses of dorsal horn neurons elicited by repetitive C-fiber stimulation (wind-up),[8] which is an N-methyl-D-aspartate (NMDA) receptor–mediated response,[9–11] is less sensitive to intrathecal opioids.[5,12] These NMDA receptor–elicited responses have been shown to be major contributors to central hypersensitive states underlying a number of models of difficult and prolonged pain states with important implications for clinical treatment.[13]

The formalin response is a well-established model of more prolonged pain which has a clearly defined biphasic response with the second later response being associated with a peripheral inflammation and central hypersensitivity. Previously, opioids, including morphine, given as pretreatments have been shown to inhibit the behavioral response to peripherally injected formalin.[14–18] The results of these behavioral studies are in agreement with electrophysiologic studies using intrathecal D-Ala-Gly-MePHe-Gly-ol (DAGOL), a selective μ-opioid, on the formalin response.[19–20] The clear and reproducible biphasic nature of the formalin re-

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sponse provides a suitable model for the study of the possible advantages of preemptive analgesia as compared with postadministration, and a recent behavioral study has addressed this issue.[18] Preadministered intrathecal morphine dose-dependently inhibited the second phase of the response. Administration of morphine after the first phase of the formalin response inhibited the second phase of the response, with no significant difference between the effect of pre- and postadministered morphine on the second phase of the formalin response being observed.[18] This is suggestive of no advantage of preemptive spinal morphine in this animal model of inflammatory pain. We address these problems using electrophysiologic techniques in the anesthetized rat where the presence of anesthesia in this animal model should replicate conditions in clinical surgery.

In the first part of this study we studied the ability of morphine to inhibit the acute electrically evoked C-fiber and Aβ-fiber responses of the dorsal horn neurons. Previous studies have not separately quantified the effect of morphine on the wind-up of dorsal horn neurons from its effect on the overall C-fiber evoked response. In this study the effects of morphine on the input and wind-up component of the C-fiber evoked response were analyzed. Since wind-up is frequency dependent, requiring higher frequencies for its induction,[9] two frequencies of stimulation (0.5 and 0.1 Hz) were used to compare opioid effects on the same neurons in the presence and absence of wind-up.

In the second part of this study we investigated preemptive approaches to opioid analgesia. The question asked was whether we could provide basic electrophysiologic evidence for doses of preadministered μ-opioid agonist morphine providing a greater degree of inhibition than the same doses of postadministered morphine. The effect of pre- and postadministration of intrathecal morphine on the second phase of the formalin response was studied. To allow for direct comparison, the timing of the pre- and postadministration of morphine was identical to a recent behavioral study,[18] with the postadministration being early after the formalin injection. Clinical studies on preemptive strategies tend to compare them to early posttreatments, which does not necessarily address the ability of opioids to inhibit fully established pain states such as those occurring at later times after surgery. To address this we examined the dose response relationship for the effects of another μ-opioid agonist DAGOL, on the formalin response when administered either as a pretreatment or as a late postadministration given when the second phase of the response had fully developed. Rapid inhibitions were required for this latter experiment so DAGOL, which has an identical pharmacology to morphine but a faster onset of action was used.[5,6]

Materials and Methods

The techniques used have been previously described.[9] Sprague-Dawley rats (200–250 g) were anesthetized with 2–3% halothane in a 66% N_2O and 33% O_2 mixture. A laminectomy was performed exposing segments L1–L3, anesthesia was maintained with 1.5% halothane. The experimental conditions followed the guidelines on animal care issued by the International Association for the Study of Pain and the experimental aims and techniques were approved by the Home Office (U.K.). Parylene-coated tungsten electrodes were used to record extracellularly the responses of convergent dorsal horn neurons which responded to noxious pinch and innocuous touch. Single neuronal responses to transcutaneous electrical stimulation (2-ms–wide pulses, 0.5 and 0.1 Hz) of the receptive field over the toes of the hindlimb were recorded. Data were recorded and analyzed with a CED 1401 interface (Cambridge Electronic Design, Cambridge, U.K.) coupled to a personal computer, and poststimulus histograms were compiled for the quantification of the responses.

All recorded dorsal horn neurons were located at depths between 600 and 800 μm. The electrically evoked responses were separated on the basis of threshold and latency of response. The Aβ-fiber evoked responses were taken as the action potentials recorded 0–20 ms after the electrical stimulus and C-fiber evoked responses as the action potentials recorded 90–300 ms after the electrical stimulus. The remaining neuronal response, occurring as the cells exhibited wind-up recorded 300–800 ms after the stimulus, was taken as the postdischarge of the neuron. Wind-up was calculated as the difference between the total number of action potentials at C-fiber latencies (90 and 800 ms) produced by the train of 16 stimuli and the input. The input response was calculated as the number of action potentials produced by the first stimulation multiplied by the total number of stimuli (16).

After three stable C-fiber control responses with less than 10% variation, the effect of intrathecal morphine (5 and 50 μg) on the electrically evoked responses of the dorsal horn neurons (n = 10), stimulated at a frequencies of 0.1 or 0.5 Hz, was studied at 10-min in-

tervals for 40 min postmorphine. Every cell was studied at both frequencies. The maximal effects of morphine used for the analysis were observed at 30–40 min. Any effects of morphine were reversed with intrathecal administration of 20 μg naloxone, the μ-opioid receptor antagonist.

In the second series of experiments in a different group of animals, after three stable C-fiber control responses, formalin (50 μl of 5% formaldehyde in saline) was injected subcutaneously into the toe over which the receptive field was present. The response to formalin was biphasic and quantified, with the first 10 min of firing taken as the first phase and the remaining 50 min of firing was taken as the second phase. The response to formalin in the absence of an intrathecal injection was taken as the control formalin response. The effect of intrathecal morphine (0.0025–0.25 μg) given either as a preadministration (n = 24) (15 min before the peripheral injection of formalin) or a postadministration (n = 21) (9 min after the peripheral injection of formalin) on the second phase of the formalin response was studied. Overall, five or six neurons were tested per dose.

In the third series of experiments the effect of pre- (n = 20) and postadministration (n = 24) of the μ-opioid agonist DAGOL (0.001–5 μg) on the second phase of the formalin response was studied. Again, five or six neurons were tested per dose. DAGOL was either given as a preadministration 20 min before the injection of formalin or during the second phase of the formalin response as a postadministration (30 min after the injection of formalin). DAGOL was used for these studies since the time to maximal effects (<15 min) after intrathecal injection in this model is much more rapid than that of morphine.[5,6] This allowed us to gauge the full extent of opioid inhibitions with a late posttreatment before the end of the formalin response at 1 h.

A control intrathecal injection of saline has previously been shown not to influence the responses of convergent dorsal horn neurons,[5,6] and therefore to restrict the number of animals used in this study these control experiments were not repeated.

The electrically evoked responses were expressed as mean maximal percentage changes in the control predrug responses and statistical analysis used analysis of variance and Fisher's *post hoc* tests where applicable. The results of the formalin studies were expressed as percentage inhibition of the control responses for the pretreatment and early posttreatment studies and as percentage inhibition of the predrug formalin responses for the late posttreatment studies. The results were expressed as means ± standard errors. Statistical analysis of these results used the two-tailed unpaired *t* test.

Results

With 0.5-Hz electrical stimulation, the C-fiber evoked response was dose-relatedly inhibited by 5 and 50 μg intrathecal morphine (table 1). The Aβ-fiber evoked response was only inhibited to a minor extent by 50 μg morphine (table 1). Intrathecal morphine (5 and 50 μg) dose-relatedly inhibited the C-fiber evoked response produced at 0.1 Hz, with the Aβ-fiber evoked response only being inhibited by 50 μg morphine (table 1).

The components of the C-fiber response were separately analyzed as input and wind-up. Wind-up produced with 0.5-Hz stimulation, was quantified as 292 ± 65 action potentials. With the smaller dose of intrathecal morphine (5 μg) there was a tendency for the input to be inhibited to a greater degree than the wind-up (table 2). The effect of 5 μg morphine on the electrically evoked wind-up, elicited at a frequency of 0.5 Hz, of a single dorsal horn neuron is shown in figure 1, illustrating how the wind-up response of the neuron breaks through the morphine inhibition. With the larger dose of morphine (50 μg) equal inhibitions of the input and wind-up of the dorsal horn neurons were now observed (table 2). The effects of intrathecal morphine on the electrically evoked responses of the dorsal horn

Table 1. Percent Inhibition of Control Response by Morphine

Morphine Dose (μg)	C-Fiber Stimulation Rate (%)		Aβ-Fiber Simulation Rate (%)	
	0.1 Hz	0.5 Hz	0.1 Hz	0.5 Hz
5	44 ± 10*	24 ± 11*	5 ± 13	18 ± 10
50	84 ± 11†	80 ± 10†	40 ± 4*	37 ± 9*

The mean maximal effect of intrathecal morphine on the C-fiber and Aβ-fiber-evoked responses of dorsal horn neurons elicited at two frequencies (0.1 and 0.5 Hz). The C-fiber-evoked responses elicited at 0.1 and 0.5 Hz were dose-relatedly inhibited by morphine ($F_{5,45}$ = 3.108). At both frequencies of stimulation, the Aβ-fiber-evoked response of the dorsal horn neurons was considerably less sensitive to intrathecal morphine and was only partly inhibited by 50 μg morphine ($F_{5,50}$ = 2.987). Results are expressed as percentage inhibition of the control response ± SEM. Statistical analysis, comparing the effects of morphine to the control responses, used ANOVA and Fisher's *post hoc* tests where applicable.

* $P \leq 0.05$.

† $P \leq 0.0001$.

Table 2. Percent Inhibition of Mean Maximal Effect of Control Response by Intrathecal Morphine

Morphine Dose	Wind-up Stimulation Rate		Input Stimulation Rate	
	0.1 Hz	0.5 Hz	0.1 Hz	0.5 Hz
5 μg	62 ± 10*	35 ± 9†	50 ± 12†	62 ± 7†
50 μg	82 ± 10‡	88 ± 5‡	87 ± 5*	81 ± 5*

The mean maximal effect of intrathecal morphine on the input and wind-up responses of dorsal horn neurons elicited at two frequencies (0.1 and 0.5 Hz). Wind-up and input ($F_{5,54}$ = 11.408 and $F_{5,46}$ = 3.322, respectively) elicited at 0.1 and 0.5 Hz were dose-relatedly inhibited by morphine. There was no significant difference between the degree of input elicited at the two frequencies or between the dose-related inhibitions of the input, elicited by these frequencies, by morphine. However, significantly lower levels of wind-up were elicited by 0.1-Hz stimulation as compared to 0.5-Hz stimulation ($F_{5,54}$ = 11.408, $P \leq 0.0001$). In addition, the residual wind-up elicited at 0.1-Hz stimulation was more sensitive to the smaller dose of morphine than the wind-up elicited at 0.5-Hz stimulation ($F_{5,54}$ = 11.408, $P \leq 0.05$). Results are expressed as percentage inhibition of the control response ± SEM. Statistical analysis, comparing the effects of morphine to the control responses, used ANOVA and Fisher's *post hoc* tests where applicable.

* $P \leq 0.001$.

† $P \leq 0.05$.

‡ $P \leq 0.0001$.

neurons were fully reversed, within 30 min, by intrathecal administration of the opioid receptor antagonist naloxone (20 μg).

The low frequency of stimulation (0.1 Hz) caused little or no wind-up (93 ± 18 action potentials), which was significantly lower than that elicited with 0.5-Hz stimulation ($F_{5,54}$ = 11.408, $P \leq 0.0001$, analysis of variance and Fisher's *post hoc* test). This residual wind-up was clearly inhibited by both 5 and 50 μg intrathecal morphine (table 2). In addition, this residual wind-up elicited at the low frequency was more sensitive, to the small dose of morphine, than the wind-up elicited with the high frequency of stimulation ($F_{5,54}$ = 11.408, $P \leq 0.05$, analysis of variance and Fisher's *post hoc* test). The input response elicited at 0.1 Hz was significantly inhibited by both 5 and 50 μg intrathecal morphine (table 2), with no significant difference between the effects of morphine on the input elicited at 0.1 and 0.5 Hz being observed. A typical response of a single dorsal horn neuron stimulated at 0.1 Hz is shown in figure 1 where the response can be seen to remain constant for the duration of the train of stimuli. In the presence of 5 μg morphine this response of the dorsal horn neuron is further reduced for the duration of the train of stimuli. Again all the effects of intrathecal morphine were reversed by 20 μg naloxone. Overall wind-up but not input was influenced by the frequency of stimulation. The frequency of stimulation did not alter the inhibition of the input by morphine, whereas significant differences were observed for wind-up. The difference between the effects of 5 μg morphine on the C-fiber responses at the two frequencies may be explained by the contribution of wind-up, itself poorly responsive to opioids, to the overall C-fiber evoked response. Thus, the ability of morphine to inhibit the C-fiber responses of the same neurons critically depends on whether wind-up is present or not but dose escalation overcomes the negative effects of wind-up.

The control first- and second-phase response of the dorsal horn neurons to the peripheral injection of formalin were 5,669 ± 1,295 action potentials and 23,516 ± 7,803 action potentials (n = 11). The effect of intrathecal preadministration and postadministration of morphine (0.0025, 0.01, 0.025, and 0.25 μg) on the dorsal horn neuronal response to a peripheral injection of formalin was studied. The second phase of the formalin response was inhibited in a dose-related manner by preadministered morphine (fig. 2). The highest dose of preadministered morphine studied (0.25 μg) significantly inhibited the second phase of the formalin response (61 ± 16%, $P \leq 0.05$). The second phase of the formalin response was inhibited by

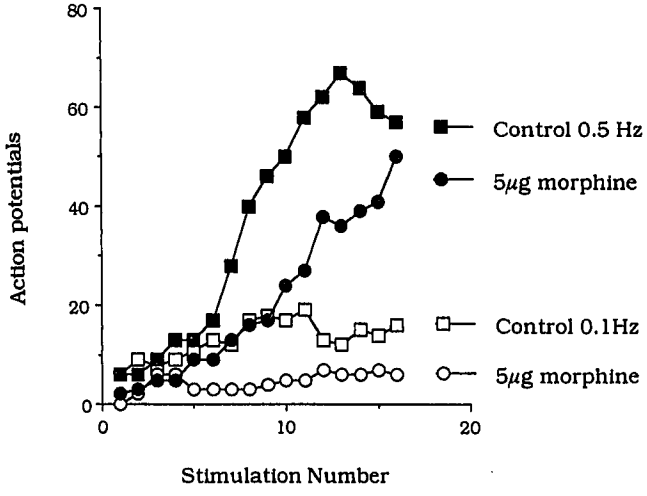

Fig. 1. The effect of intrathecal administration of 5 μg morphine on the wind-up of a single dorsal horn neuron. Wind-up responses were elicited by electrical stimulation at a frequencies of 0.5 and 0.1 Hz. The control wind-up responses (in the absence of morphine) to stimulation at frequencies of 0.5 and 0.1 Hz illustrate the minimal wind-up produced at 0.1 Hz. In the presence of 5 μg morphine the response of the same neuron to stimulation at 0.1 Hz was almost abolished, whereas the wind-up produced at 0.5 Hz overcame the opioid inhibition.

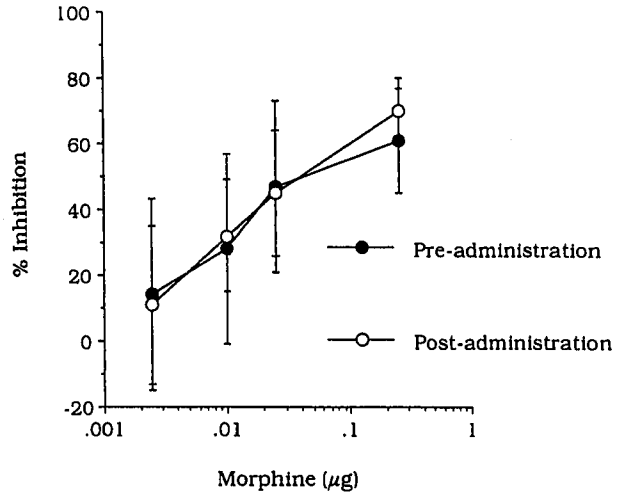

Fig. 2. Dose–response curves showing the effect of intrathecal morphine given as a preadministration and early (9 min) postadministration on the second phase of the formalin response. Both pre- and postadministered morphine dose-relatedly inhibited the second phase of the response, in a similar manner. The effects of both pre- and postadministration of morphine (0.25 μg) were significantly different from the control responses but not from each other.

early (9 min postformalin) postadministered morphine in a dose-related manner (fig. 2), with 0.25 μg morphine significantly and equally inhibiting the second phase of the response (70 ± 10%, $P \leq 0.05$). The morphine dose–response curve for postadministered morphine was identical to that observed with preadministered morphine (fig. 2) showing that these timings did not influence the ability of morphine to inhibit the second phase of the formalin response.

The effect of pre- *versus* a late (30 min) postadministration of DAGOL on the second phase of the formalin response was studied. Preadministered DAGOL dose-relatedly inhibited the second phase of the formalin response (fig. 3) with 0.25 μg DAGOL significantly inhibiting the response (68 ± 21% inhibition, $P \leq 0.01$) and 5 μg DAGOL virtually abolishing it (98 ± 1% inhibition, $P \leq 0.001$). However, the second phase of the formalin response was less sensitive to the inhibitory effects of the smaller doses of DAGOL when administered once the second phase of the formalin response had commenced (fig. 3). For example, 0.25 μg DAGOL did not significantly inhibit the response in clear contrast to the inhibitory effect of this dose when given as a preadministration. One μg administered after the formalin produced a 64 ± 16% inhibition ($P \leq 0.05$). However, postadministration of the largest dose of DAGOL (5 μg) significantly inhibited the second phase of the response (90 ± 8% inhibition, $P \leq 0.001$), a comparable inhibition to that observed with preadministration of the same dose.

Discussion

The selective inhibitory effects of morphine on the C-fiber responses of dorsal horn neurons compared to the partial effects on the Aβ-fiber evoked responses of these neurons are in agreement with a previous electrophysiologic study.[5]

Analysis of the C-fiber evoked response elicited at the two stimulation frequencies showed that wind-up of the dorsal horn neurons is poorly sensitive to low doses of morphine, but is inhibited by larger doses of the opioid. The input onto the neurons was always sensitive to morphine, irrespective of the frequency.

The differential effects of small *versus* large doses of morphine on the input and wind-up may be explained by the location of the opioid receptors. Seventy percent of μ-opioid receptors are located presynaptically[21] and it is likely that submaximal doses of morphine (5 μg) act mainly at these presynaptic sites.[22] The presynaptic reduction in transmitter release from C-fiber afferent fibers will reduce the input onto the dorsal horn neurons. Low dose morphine (5 μg) reduced but did not

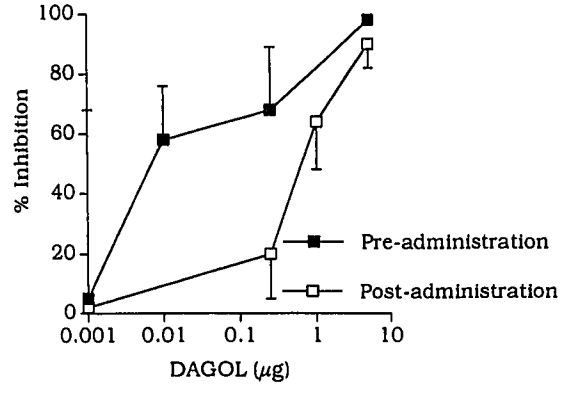

Fig. 3. Dose–response curves showing the effect of intrathecal DAGOL on the second phase of the formalin response. The responses were highly sensitive to preadministered D-Ala-Gly-MePHe-Gly-ol (DAGOL), whereas with late (30 min) postadministration, the smaller doses of DAGOL were less effective at inhibiting the second phase of the response. The highest dose of DAGOL (5 μg) given as a postadministration inhibited the second phase of the response to the same extent as did preadministration, leading to a nonparallel shift in the dose–response curve.

completely block the input onto the dorsal horn neurons, in agreement with studies which have shown that opioids reduce but do not completely abolish noxious evoked release of substance P[23-25] and glutamate[26] into the dorsal horn of the spinal cord. The reduced input onto the dorsal horn neurons may still be able to activate post synaptic excitatory receptors. With sufficient depolarization of the post synaptic membrane the voltage dependent magnesium block of the NMDA receptor complex will be removed, leading to the activation of the NMDA receptor and the induction of wind-up. Large doses of morphine will act at both pre- and postsynaptic opioid receptors,[22] and the additional potential postsynaptic action of large doses of morphine will counter NMDA receptor activation produced by the residual input. Therefore the combined activation of pre- and postsynaptic receptors by large doses of morphine results in a potent inhibition of wind-up. Importantly, the relative insensitivity of NMDA receptor–mediated wind-up to low doses of morphine as compared to the steady C-fiber evoked input response can be overcome by larger doses of morphine.

The second part of the study investigated the effects of pre- and postadministered morphine on the second phase of the formalin response. An early postadministration of the large dose of morphine produced an identical inhibition of the second phase of the formalin response compared to preadministration of the same dose of morphine. Thus the timing of the administration of intrathecal morphine does not alter the ability of morphine to inhibit the second phase of the formalin response, in complete agreement with a recent behavioral study.[18] By contrast, the second phase of the formalin response was shown to be less sensitive to smaller doses of DAGOL administered during the second phase of the formalin response (late postadministration) as compared to preadministration of the same doses of DAGOL. As with wind-up, the largest dose of DAGOL administered during the formalin response was as effective at inhibiting the second phase of the formalin response as the same dose of preadministered DAGOL. In the present study and the behavioral study of Yamamoto and Yaksh[18] postadministered morphine was administered at the end of the first phase, before the second phase of the response and therefore before NMDA receptor activation had commenced. However, postadministered DAGOL was administered during the second phase of the formalin response, after NMDA receptor activation had occurred. Since we show that small doses of μ-opioid agonists are less effective at inhibiting NMDA receptor–mediated events such as wind-up, the ongoing NMDA receptor mediation of the second phase of the formalin response may in part explain the decreased ability of small doses of DAGOL, administered during the second phase, to inhibit the second phase of the formalin response. Likewise the ability of large doses of μ-opioid agonists to overcome NMDA receptor–mediated events is reflected in the results of both the wind-up and formalin studies with late postadministration of DAGOL. The reduced sensitivity of NMDA receptor–mediated events to opioid inhibitions has been observed in a number of electrophysiologic studies where NMDA receptor activation maintains the enhanced responses to noxious stimulation.[1,5,7] However, this has not always been observed in behavioral studies.[18]

The greater sensitivity of the formalin response to the opioids compared to the acute electrically evoked C-fiber response is in agreement with previous electrophysiologic[27] and behavioral studies.[2,18]

In conclusion, our study supports the hypothesis that excessive excitability, including NMDA receptor–mediated wind-up may relate to far reaching alterations in central pain processing which may contribute to the development of opioid insensitive pain.[1,28] The formalin response is due to inflammatory stimulation but the same reduction in opioid analgesia produced by nociceptive events, at least partly including NMDA receptor activation, appears to occur in a number of animal models of central and peripheral neuropathies.[29]

Recent clinical studies have addressed the relative merits of preemptive opioids *versus* posttreatment with opioids.[30-31] Although these two studies provided evidence for preemptive opioids being more effective than posttreatments, the differences between the two treatments were not as marked as one might have expected. A number of factors could be critical in determining the outcome of these studies, including the timing of postadministered opioids, the chosen doses of opioids and the time point of outcome testing. In fact, the present study clearly shows that the first two factors are of prime importance. We observed that the reduced ability of late opioid posttreatment to inhibit the formalin response could be overcome by either increasing the dose or by earlier posttreatment. Thus in clinical studies, any difference between preemptive and posttreatments could be masked by these variables. Moderate doses given *post hoc* would overcome the reduced sensitivity to opioids. It is also important to note that in the clinical studies the opioid was given

very early, either after the incision or at closure, equivalent to an early posttreatment.[30,31] The effect of preemptive analgesia may never be satisfactorily resolved since studies using late posttreatments would require the withholding of analgesics in the postoperative period which may compromise the clinical condition of the patient. A final factor to be considered is the induction of immediate early genes, such as c-*fos,* as a result of noxious inputs including surgery and inflammation, which in the longer term may influence the transmission and modulation of pain and therefore the interpretation of preemptive analgesia.[32]

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