

## Effect of Spinal Morphine after Long-term Potentiation of Wide Dynamic Range Neurones in the Rat

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**Background:** Studies have shown that long-term increase in the excitability of single wide dynamic range neurones in the spinal dorsal horn of rats may be induced after tetanic stimulation to the sciatic nerve. This sensory event is possibly an *in vivo* counterpart of long-term potentiation, described in the brain. This study investigated whether this phenomenon occurs in the halothane-anesthetized rat and whether the antinociceptive effects of spinally administered morphine are altered when tested on the enhanced activity.

**Methods:** Single unit extracellular recordings were made in three different groups of halothane-anesthetized rats ( $n = 6$  in each group). In group 1, the evoked neuronal responses of wide dynamic range neurones by a single electrical stimulus to the peripheral nerve were recorded every 4 min, for 1 h before (baseline) and for 3 h after brief high-frequency conditioning stimulation of the sciatic nerve. In group 2, morphine was applied onto the spinal cord after long-term potentiation had been established. Increasing concentrations of morphine were added until the C fiber-evoked responses were abolished; this was followed by naloxone reversal. In group 3, the same pro-

tolocol as in group 2 was used except a waiting period substituted for the electrical conditioning.

**Results:** The C fiber-evoked responses were significantly increased ( $P < 0.001$ ) after conditioning compared with baseline and those in control animals. Further, significantly higher concentrations of morphine ( $P = 0.008$ ) were needed to abolish the C fiber-evoked responses in tetanized animals than in control animals. Naloxone reversed the effects of morphine to the pre-drug potentiated baseline in group 2, showing that opioids do not block the maintenance of spinal long-term potentiation.

**Conclusions:** Long-term potentiation of C fiber-evoked responses also can be induced in halothane-anesthetized rats, and morphine seems to have less potency during such conditions. These data suggest that long-term potentiation-like mechanisms may underlie some forms of hyperalgesia associated with a reduced effect of morphine. (Key words: Central sensitization; pain; spinal cord.)

IN 1983, evidence was provided for a central component of postinjury pain hypersensitivity,<sup>1</sup> and later it was shown that electrical and chemical conditioning stimulation could facilitate the heterosynaptic flexor reflex of spinalized rats for periods ranging from 3 min to  $>3$  h.<sup>2</sup> Further, spinal long-term potentiation (LTP) has been reported in *in vitro*<sup>3,4</sup> and *in vivo*<sup>5–7</sup> studies using electrical conditioning and after natural noxious stimulation.<sup>8,9</sup>

Mechanisms of chronic pain are not well understood. It is puzzling that, after an episode of acute pain, hyperalgesia and allodynia may persist for years despite perfect healing of tissue.<sup>10</sup> It is conceivable that some form of adaptive or learning process in spinal circuitry may underlie this phenomenon. It has been suggested that spinal LTP may be an important part of the more general term central sensitization,<sup>11,12</sup> the behavioral correlate of which is hyperalgesia.

All previous *in vivo* studies showing spinal LTP have used urethane as the anesthetic agent. In the current study, we wanted to investigate whether LTP of C fiber-evoked responses could be induced in halothane-anesthetized rats, as urethane is only used in animal experi-

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ments, whereas the most commonly used inhaled anesthetic agent is isoflurane in adults and halothane in children.<sup>13</sup>

A long-term increase in evoked neuronal firing over periods up to 6 h has been shown, based on the recording of single wide dynamic range (WDR) neurones in the dorsal horn of the spinal cord after tetanic electrical stimulation of the sciatic nerve.<sup>7,11</sup> The stimulation technique used to show this phenomenon has been proposed as a novel method of studying plasticity within spinal nociceptive circuits.<sup>7</sup> Thus, we thought it important to study the effect of spinally administered morphine using the same stimulation technique. In this way, we could gauge whether the effects of a spinally acting opioid were altered by the presence of enhanced excitability induced by peripheral stimulation.

## Materials and Methods

Experiments were performed on 18 male Sprague-Dawley rats (University College animal house) weighing 180–220 g. Rats were given free access to food and water; a 12-h light/dark cycle was used. All procedures described were approved by the UK Home Office. Methods were similar to those previously described.<sup>14</sup> Rats were intact, anesthesia was induced with 2.5–3.0% halothane in  $\frac{2}{3}$  N<sub>2</sub>O and  $\frac{1}{3}$  O<sub>2</sub>, and an endotracheal tube was inserted. Abolished pedal reflex and corneal reflex indicated adequate anesthesia. A temperature probe was inserted in the colon, and the core temperature was kept constant at 36.5–37.5°C by means of a heating pad with an electronic control unit. Rats were placed in a stereotaxic frame to ensure stability during electrophysiologic recordings and then given 5 ml saline subcutaneously to prevent dehydration. With the rat fixed in a prone position, laminectomies of L1 or L2 were performed to expose segments L4–L5 of the spinal cord. The vertebral column was held rigid by two clamps placed rostrally and caudally to the exposed section of the cord. The left sciatic nerve was then dissected free in the mid-thigh level where it runs between muscle compartments, and at least 1 cm of the nerve was exposed. A bipolar silver hook electrode (distance of 2 mm between hooks) was placed under the nerve immediately proximal to where it divides.<sup>7</sup> The electrodes were separated from surrounding muscles by a sheet of thick elastic plastic (Parafilm; American Can Company, Greenwich, CT). Once surgery was completed, the level of anesthesia was reduced to 1.7–2.0% halothane, which abolished the

pedal withdrawal reflex and the corneal reflex. This level of anesthesia was maintained throughout the experiment, which lasted up to 8 h, and at the end of the experiment rats were killed with an overdose of halothane.

### *In Vivo Electrophysiology*

A parylene-coated tungsten electrode was lowered into the dorsal horn of the spinal cord, and WDR neurones were isolated in the deep laminae (510–980  $\mu$ m from the surface of the cord). It was required that the response to tactile skin stimulation increase with increasing tactile force and that the response to pinch was always greater than the response to pressure to ensure that they were WDR neurones. Hence, only WDR neurones and no nociceptive specific neurones were studied. Only one cell per animal was used. Spikes observed 0–45 ms, 45–300 ms, and 300–800 ms after a stimulus were defined as A and C fiber-mediated responses and postdischarge (the firing after the main C fiber band of activity), respectively. The 2-ms rectangular pulse used as a test stimulus was given with a pulse current of 1.5 times the C fiber threshold.

During the experiment, attempts were made to ensure minimal afferent input. The nerve dissection was done with extreme care, and, while searching for a cell, only light touch was used. Then, pinch and electrical stimulation were used to examine whether the neurone was a WDR cell. The nerve was never given >10 electrical stimuli before the baseline recording started. The frequency of test stimuli was low (one stimulus every 4 min). The cell was rejected if the C fiber-evoked response was less than five spikes initially.

### *Experimental Procedure*

Eighteen rats were divided into three different groups ( $n = 6$  in each group). In group 1, WDR neurone firing to single electrical stimuli (one stimulus every 4 min) was recorded for 1 h (baseline) followed by a conditioning stimulus. After a 10-min waiting period, the neuronal firing to single electrical test stimuli was recorded for 3 h<sup>11,15</sup> in the same way as for the baseline response. The conditioning stimulation was similar to previous studies (20 trains lasting 2 s at 100 Hz given every 10 s, 0.5-ms width, at six times the C fiber threshold previously found using a 2-ms-wide pulse).<sup>7,11,15,16</sup>

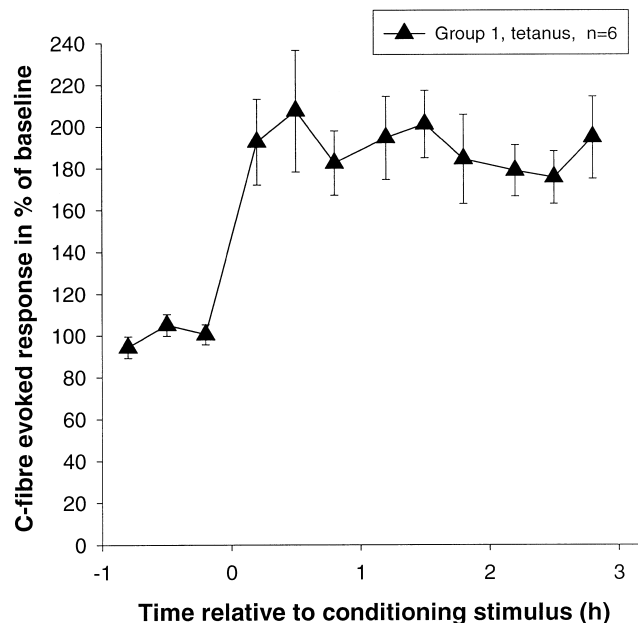
In group 2, the same procedure was used as for group 1 for 1 h after conditioning; then, morphine was applied onto the exposed surface of the dorsal horn. This is equivalent to an intrathecal injection in that the drug

was applied onto the pia below the dura that was removed to allow insertion of the electrode into the spinal cord. As the drug was applied to the surface and then left in place, this approach effectively creates a constant concentration bath. Morphine was applied in a volume of 50  $\mu\text{l}$ , to fill the segment exposed by the laminectomy, from a glass pipette. Drugs were made up in saline. Morphine was applied in increasing concentrations (0.3, 1.0, 3.0, and 10.0  $\mu\text{g}$  in 50  $\mu\text{l}$ ) until the C fiber-evoked responses were abolished. The effect of each dose was monitored for 44 min. Naloxone (5  $\mu\text{g}$  in 50  $\mu\text{l}$ ) was applied and the effect monitored for 30 min when the C fiber-mediated response was zero in four consecutive recordings. It has been shown previously that the peak effects of similar doses of morphine and naloxone are seen 40 and 20–25 min after spinal administration, respectively, so that we were at steady state when the next dose was administered.<sup>17</sup> In group 3, morphine and naloxone were applied in the same manner as in group 2, but the conditioning stimulation was substituted with a waiting period (3 min, 20 s). The thresholds for A and C fiber-evoked responses were measured and controlled once every hour in group 1 but not in groups 2 and 3, as morphine was applied. It has been shown that neither the receptive fields nor the spontaneous activity of the WDR neurones is altered after this conditioning stimulation<sup>7,11</sup>; hence these parameters were not measured here.

#### Statistical Analysis

The effect of the conditioning stimulation was evaluated by one- and two-way analysis of variance (ANOVA; repeated-measures design, with the Hyunh-Feldt correction of degrees of freedom used as necessary). The mean of the responses to every four consecutive stimuli calculated represents one value.<sup>7</sup> This gave three values in the baseline period and a minimum of nine values after the conditioning stimulus in all three groups. All statistical calculations were performed using raw data.

Comparison of the overall effect of morphine between groups 2 and 3 was done with multiple regression analysis (with the groups as dummy variables, drug effect as dependent variable, and the dose of morphine as the independent variable) after transformation to percent values. The average response of the first hour after conditioning was set to 100% (baseline 2). Thresholds for activating A and C fibers were compared using Student *t* test.



**Fig. 1.** C fiber-evoked firing responses (mean  $\pm$  SEM in percent of baseline,  $n = 6$ ). The responses were significantly increased for 3 h after conditioning ( $P < 0.001$ ). The values shown are based on the average of four consecutively recorded responses (100% = 7.5 action potentials).

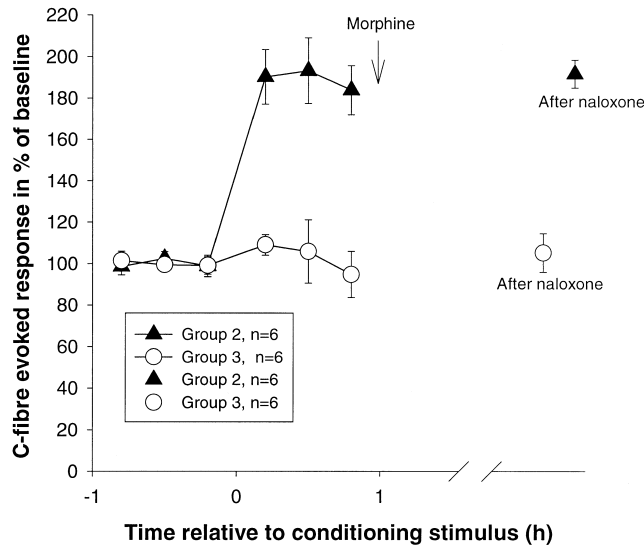
## Results

The neurones in all three groups ( $n = 6$  in each group) were located at comparable depths in the dorsal horn (group 1,  $682 \pm 47 \mu\text{m}$ ; group 2,  $748 \pm 68 \mu\text{m}$ ; and group 3,  $772 \pm 73 \mu\text{m}$  from the surface of the cord). Thresholds for activating A and C fibers were not significantly different among the three groups at the start of the experiment (A fibers,  $0.45 \pm 0.06$  vs.  $0.48 \pm 0.01$  vs.  $0.47 \pm 0.01$  mA; C fibers,  $1.24 \pm 0.11$  vs.  $1.26 \pm 0.08$  vs.  $1.27 \pm 0.09$  mA, respectively). Further, the thresholds were not significantly changed 10 min after conditioning, and in group 1 they were stable throughout the experiment (3 h, data not shown).

#### Effect of Conditioning Stimulation

After the conditioning, there was a significant increase in C fiber-induced firing responses of single WDR neurones compared with the baseline response ( $P < 0.001$ , one-way ANOVA repeated-measures design,  $n = 6$ ; fig. 1) and that in control animals ( $P < 0.001$ , ANOVA repeated-measures design, two-way interaction group  $\times$  time, six levels,  $n = 12$ ; figs. 2, 3A, and 3B). Although there was a large increase in C fiber-evoked firing after conditioning, the pattern of the response to the stimulus was

## MORPHINE AND SPINAL LONG-TERM POTENTIATION



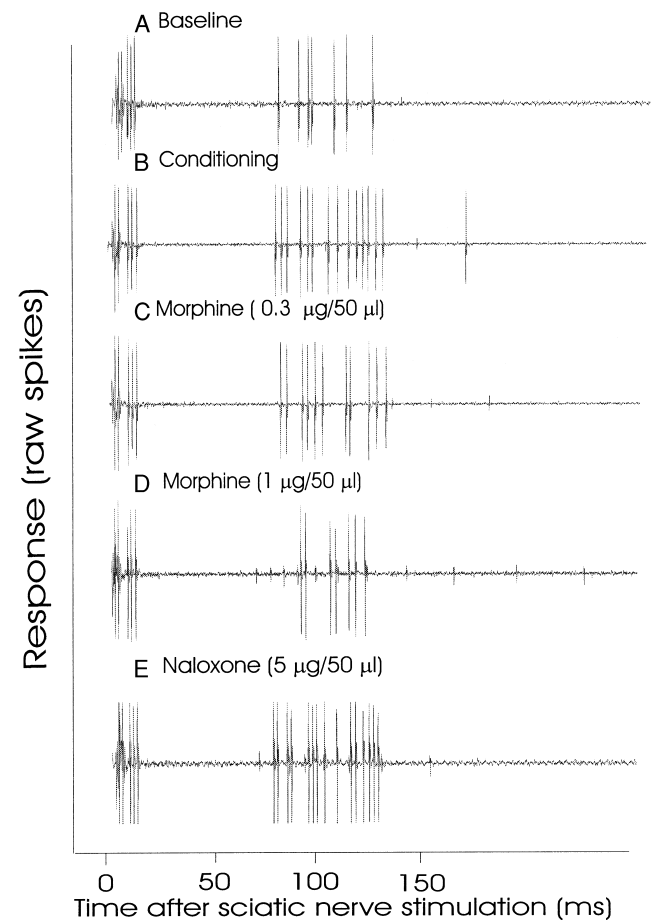
**Fig. 2.** C fiber-evoked firing responses (mean  $\pm$  SEM in percent of baseline). The responses were significantly increased for 1 h ( $P < 0.001$ ) after conditioning compared with baseline and those in control animals. The values shown are based on the average of four consecutive recorded responses (100% = 7.8 and 11.5 action potentials in groups 2 and 3, respectively). Morphine was applied 1 h after tetanization (group 2) or a waiting period (group 3). The time from morphine was applied until the C fiber-evoked response was zero varied from cell to cell (2–4 h). Naloxone, however, reversed the C fiber-mediated response to the control level in group 3 and to the LTP level in group 2.

unchanged (fig. 3). A comparison between tetanized (group 2) and control animals (group 3) was made for the 1-h period after conditioning before the application of morphine. Naloxone, however, reversed the effect of morphine to either the control or the LTP level (see subsequent section) in the appropriate group, indicating that the difference produced by the conditioning stimulation outlasted the experiments (3–5 h; fig. 2). There was no significant change (A fiber-induced responses,  $100 \pm 4.5\%$  vs.  $107 \pm 15\%$ ; postdischarge,  $100 \pm 39\%$  vs.  $79 \pm 41\%$ ;  $P = 0.18$  and  $0.26$ , respectively, one-way ANOVA repeated-measures design, 12 levels,  $n = 6$ ) or difference between groups 2 and 3 (A fiber-induced responses,  $95 \pm 8\%$  vs.  $134 \pm 37\%$ ; postdischarge,  $80 \pm 24\%$  vs.  $77 \pm 25\%$ ,  $P = 0.59$  and  $0.68$ , respectively, two-way ANOVA repeated-measures design, six levels,  $n = 12$ ) in either A fiber-induced responses or after discharge after conditioning.

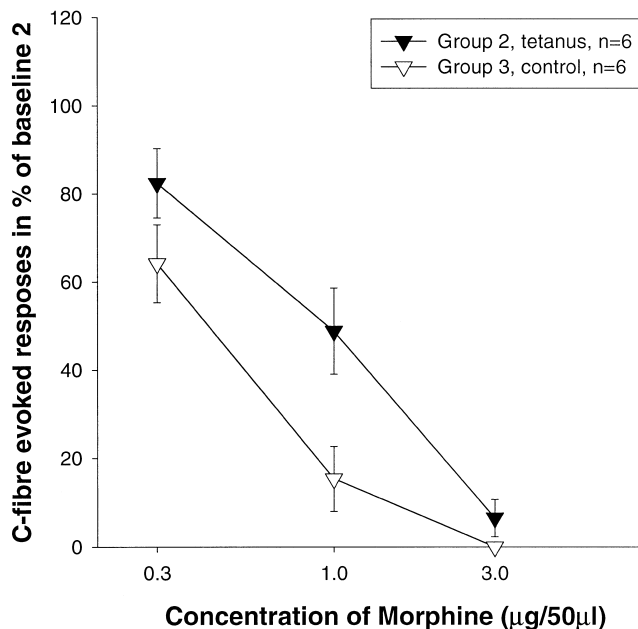
#### Pharmacology

In groups 2 and 3, the effect of increasing concentrations of spinally administered morphine was studied from 1 h

after conditioning stimulation (group 2) or a waiting period (group 3) until the C fiber-evoked response was abolished. All 12 cells responded in a dose-related manner, and inhibitions were reversed by naloxone. These effects were selective for the C fiber-evoked responses (fig. 3). The time courses for the effect of morphine in both groups were not significantly different ( $P = 0.56$ , two-way ANOVA repeated-measures design, nine levels,  $n = 12$ ). The maximum morphine-induced inhibition was reached at similar time points in the two groups ( $31 \pm 4$  vs.  $33 \pm 4$  min after  $0.3 \mu\text{g}/50 \mu\text{l}$ ;  $37 \pm 4$  vs.  $36 \pm 5$  min after  $1 \mu\text{g}/50 \mu\text{l}$ ; and  $38 \pm 4$  vs.  $36 \pm 4$  min after  $3 \mu\text{g}/50 \mu\text{l}$  in groups 2 and 3,



**Fig. 3.** Poststimulus diagram showing the raw spike responses of a characteristic cell in the tetanus group (group 2). (A) Response to the first test stimulus in the baseline (five A fiber- and seven C fiber-mediated spikes). (B) Response to the first test stimulus after conditioning (5 A fiber- and 15 C fiber-mediated spikes). (C) Response at steady state of  $0.3 \mu\text{g}$  morphine in  $50 \mu\text{l}$  (5 A fiber- and 12 C fiber-mediated spikes). (D) Response at steady state of  $1 \mu\text{g}$  morphine in  $50 \mu\text{l}$  (5 A fiber- and seven C fiber-mediated spikes). (E) Response at steady state of  $5 \mu\text{g}$  naloxone in  $50 \mu\text{l}$  (5 A fiber- and 15 C fiber-mediated spikes).



**Fig. 4.** The effect of three concentrations of spinally administered morphine on the C fiber-evoked firing responses (mean  $\pm$  SEM in percent of baseline 2, the first hour after conditioning [group 2] or a waiting period [group 3]). Each concentration was left on the cord for at least 44 min before being replaced by a higher one. The values shown are the maximum inhibitory effects produced by each concentration of morphine at steady state based on the average of four consecutively recorded responses (100% = 14.0 and 11.25 action potentials in groups 2 and 3, respectively). The overall effect of morphine was significantly different between the two groups ( $P = 0.008$ ).

respectively,  $n = 12$ ). In two of six cells in group 3 (control animals), the C fiber-evoked response was abolished already during the second concentration of morphine (1  $\mu\text{g}$  in 50  $\mu\text{l}$ ), whereas the other four neurones needed the third (3  $\mu\text{g}$  in 50  $\mu\text{l}$ ). In group 2 (tetanized animals), the C fiber response was reduced to zero in four cells during this concentration (3  $\mu\text{g}$  in 50  $\mu\text{l}$ ), whereas two neurones needed the highest (10  $\mu\text{g}$  in 50  $\mu\text{l}$ ). Comparing the maximum inhibitory effect of single concentrations in the two groups gave significant differences at one concentration (1  $\mu\text{g}/50 \mu\text{l}$ ,  $P = 0.02$ , Mann-Whitney U test,  $n = 12$ ; fig. 4). The overall effect of the three lowest concentrations of morphine (0.3, 1.0, and 3.0  $\mu\text{g}$  in 50  $\mu\text{l}$ ) was also significantly different in the two groups ( $P = 0.008$ , multiple regression analysis, drug effect as the dependent variable, different concentrations of morphine as the independent variable, and groups as dummy variables,  $n = 12$ ; fig. 4). Naloxone (5  $\mu\text{g}/50 \mu\text{l}$ ) reversed the morphine-related inhibitions of the C fiber response to the predrug control

values (105  $\pm$  9% of the control, group 3) and LTP values (97  $\pm$  9% of the postconditioning level, group 2; fig. 2).

## Discussion

We report for the first time LTP of the C fiber-evoked responses of WDR neurones in intact halothane-anesthetized rats. This suggests that similar long-term enhancement of C fiber-evoked activity can occur during and after surgery with volatile anesthesia, such as with isoflurane or halothane in humans. We found no LTP of the A fiber-evoked response in contrast to the results from a similar previous study using urethane anesthesia.<sup>7</sup> This is likely because of a muscle relaxant effect of halothane and nitrous oxide.<sup>18,19</sup> In urethane-anesthetized rats receiving neuromuscular blockade (pancuronium bromide), eliminating tetanic muscular contractions, no A fiber LTP was found.<sup>11</sup> Thus, although an LTP of the C fiber-evoked responses is observed with both forms of anesthesia, the combination of nitrous oxide and halothane, akin to that used during surgical procedures in humans, protects against A fiber LTP, which appears to be elicited indirectly from muscle afferents. This may result from the muscle relaxant properties of this anesthetic combination.

The exact location and the nature of potential cellular and synaptic changes underlying the increased excitability of WDR neurones cannot be decided based on this study. In the hippocampus, LTP is associated with many synaptic and intracellular changes.<sup>20</sup> It is possible that the LTP-like phenomenon observed in this study may be associated with similar changes leading to increased excitability of circuits between the primary afferent neurone and the WDR cell. Furthermore, altered states of descending or local inhibitory systems could also contribute. Thus, C fiber LTP and LTP of the postdischarge of the neurones have been shown recently in spinalized animals with a peripheral nerve block,<sup>9,16</sup> indicating that the principal mechanisms are located in the spinal cord, most likely in the dorsal horn, and that LTP of the postdischarge is controlled during normal conditions by descending pathways.

Morphine was less effective against the potentiated responses. It could be argued that this resulted from either neuronal mechanisms or was attributable to non-neuronal events, such as increased spinal blood flow produced by the tetanic stimulation. The former is much more likely. If spinal blood flow and consequently the clearance of spinal morphine increased, then the time

courses of the effects of morphine should differ between the two groups; however, they were exactly the same for both groups. Furthermore, the magnitude of the naloxone reversal was greater in the tetanized group (regarding number of evoked spikes restored). This is the opposite of what would have been predicted if there was increased clearance of the antagonist, more liposoluble than morphine itself. In this regard, spinally administered naloxone immediately reversed the effects of spinally administered morphine, showing that there was no systemic distribution contributing to the observed opioid actions. Furthermore, after inflammation, where the peripheral changes also may increase spinal blood flow, morphine, by the same route, is more effective, not less.<sup>21</sup> The addition of morphine to the exposed surface of the spinal cord creates a constant concentration bath that mitigates against any changes in clearance produced by changes in blood flow. Finally, this study required long recordings of neurones during carefully controlled conditions. Although increases in blood flow may arise from the increased blood pressure produced by the stimulation,<sup>11</sup> the latter decreases rapidly after the tetanic stimulus; however, the altered effectiveness of morphine was still seen 3–4 h later. In a study examining spinal flow after a similar form of tetanic stimulation in rats, the increased spinal blood flow, entirely explicable by the increased blood pressure, lasted only 1 min.<sup>22</sup> Thus, we believe that the substrates for C fiber LTP and morphine analgesia are spinal neuronal systems.

Thus, neuronal mechanisms likely underlie the finding that spinally administered morphine inhibited the C fiber-evoked response significantly less in tetanized animals, although in the LTP group (group 2), increasing the dose led to complete inhibitions (see Results). Morphine acts at presynaptic and postsynaptic opiate receptors in the spinal cord, but the proportion of presynaptic opiate receptors on fine afferent fibers is greater, being 75% of the total  $\mu$ -receptor, in the rat spinal cervical cord. Although this has not been studied in the lumbar spinal cord, there seem to be no rostrocaudal changes in total opioid receptor number.<sup>23</sup> Thus, with a given dose of morphine, a presynaptic inhibition of transmitter release likely predominates over a postsynaptic inhibition of neuronal activity. Consequently, presynaptic and postsynaptic mechanisms in the inhibitory effects of morphine at the lumbar spinal level have differential dose requirements, with higher doses being required for postsynaptic inhibitions, although this study only examined spontaneous activity.<sup>24</sup> Postsynaptic excitatory receptors, such as AMPA and *N*-methyl-D-aspartate recep-

tors, are important in the induction and maintenance of C fiber LTP,<sup>11,15</sup> and therefore higher doses of the opioid might be expected to be needed to overcome the increased excitability of the postsynaptic neurones. Using evoked responses, short-term wind-up of spinal neurones is less sensitive to spinally administered morphine than the nonpotentiated baseline response,<sup>17</sup> and conditioning stimuli applied to C fibers produces an enhanced withdrawal reflex, which is also less sensitive to morphine when given after conditioning.<sup>25</sup> The reduced potency of morphine seen in these studies and the current study could therefore be a consequence of an increased activity of postsynaptic excitatory receptor systems. Altered sensitivity of opiate receptor-effector mechanisms, however, cannot be excluded. Increased release of the peptide cholecystokinin, which is an endogenous modulator of the  $\mu$ -opiate receptor, also might be involved.<sup>26</sup> Finally, the difference between the groups could be attributable to a slight difference in baseline (action potentials per stimulus: tetanus,  $14.0 \pm 0.96$ ; control,  $11.25 \pm 0.88$ ). This is unlikely, however, because no significant correlation between the degree of inhibition produced by a given dose of morphine and the magnitude of the premorphine C fiber response has been seen.<sup>27</sup>

It is noteworthy that naloxone reversed the complete morphine inhibition of the C fiber response exactly to the postconditioning LTP level (fig. 2), not to the original control level, showing that the inhibition of evoked activity induced by morphine does not alter the maintenance of the increased activity. It has been reported recently that this enhanced C fiber response is, by contrast, restored to pre-LTP level after pretreatment with an *N*-methyl-D-aspartate receptor antagonist (D-AP5).<sup>15</sup> This suggests that AMPA and *N*-methyl-D-aspartate receptors are important in the expression of LTP in WDR neurones, although it is not known whether these receptors participate in the maintenance of excitability. In the hippocampus, activation of AMPA and *N*-methyl-D-aspartate receptors induces LTP. Consequently, the influx of calcium ions through the *N*-methyl-D-aspartate receptor channel is critical for stimulation of several intracellular signaling pathways that in turn are responsible for presynaptic and postsynaptic changes that maintain the enhanced response.<sup>20</sup> Our current results with morphine and naloxone indicate that spinal LTP, once induced, is not maintained by afferent nociceptive activity, the membrane potential, or the degree of excitability of the neurone, pointing toward intracellular changes produced rapidly after the tetanus. One could

speculate that *N*-methyl-D-aspartate receptor antagonists such as ketamine would have a greater effect than morphine in painful conditions in which LTP-like mechanisms are the origin of the pain state.

C fiber LTP can be induced in halothane-anesthetized rats, and the antinociceptive effect of spinally administered morphine is reduced in tetanized animals. LTP-like mechanisms may explain in part the need for higher doses of morphine to produce acceptable antinociceptive effect after episodes of severe pain.

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