Topical Opioids in Mice: Analgesia and Reversal of Tolerance by a Topical N-Methyl-D-Aspartate Antagonist

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ABSTRACT
In addition to its central actions, morphine has important peripheral effects. To examine peripheral analgesic mechanisms, we developed a topical opioid paradigm in which the tail was immersed in a dimethyl sulfoxide (DMSO) solution containing various drugs. Alone, DMSO was inactive in the tail-flick assay in mice. DMSO solutions containing morphine and peptides such as [D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO) produced a potent, dose-dependent analgesia with the radiant heat tail-flick assay. The actions of the drugs were local. Analgesia was observed only in regions of the tail exposed to the solution and not in more proximal unexposed portions of the tail. Immersion of the tail in a solution containing either ¹²⁵I-labeled morphine or ¹²⁵I-labeled DAMGO revealed no detectable uptake of radioactivity into the brain, spinal cord, or blood.

In the tail, radioactivity was limited only to the regions actually immersed in the solutions. The topical drugs potentiated systemic agents, similar to the previously established synergy between peripheral and central sites of action. Local tolerance was rapidly produced by repeated daily exposure of the tail to morphine. Topical morphine tolerance was effectively blocked by the N-methyl-D-aspartate (NMDA) antagonist MK801 given either systemically or topically but not intrathecally. The ability of a topical NMDA antagonist to block local morphine tolerance suggests that peripheral NMDA receptors mediate topical morphine tolerance. Morphine was cross-tolerant to DAMGO, but not to morphine-6β-glucuronide, implying different mechanisms of action. These observations are significant in the design and use of opioids clinically.

Morphine is a potent μ-opioid-receptor agonist with important central sites of action (Reisine and Pasternak, 1996). Peripheral mechanisms have also been reported, and their importance is becoming increasingly appreciated (Joris et al., 1987; Barber and Gottschlich, 1992; Junien and Wettstein, 1992; Stein et al., 1995). Peripheral analgesics have several potential advantages in the clinical treatment of pain, particularly the limitation of side effects such as constipation and sedation, which are typically seen with systemic administration. Given locally into the tail, morphine and other opioids are effective analgesics, working either alone peripherally or synergistically with central sites (Kolesnikov et al., 1996). In many respects, these studies are similar to clinical investigations (Joris et al., 1987; Mays et al., 1987; Dahl et al., 1990; Heard et al., 1992; Khoury et al., 1992; Raja et al., 1992; Stein, 1993; Dalsgaard et al., 1994). Peripheral mechanisms also have been implicated in systemic morphine tolerance (Kolesnikov et al., 1996). Early studies reported that systemic morphine tolerance is associated with no change in sensitivity to morphine given either spinally or supraspinally (Roerig et al., 1984). Although we also found no change in potency for spinal or supraspinal morphine after chronic morphine dosing, we did observe a profound reduction in its potency peripherally (Kolesnikov et al., 1996). In the current studies, we investigated the use of topicaly administered drugs as a means of exploring peripheral opioid actions and systemic morphine tolerance.

Materials and Methods
Male Crl:CD-1(ICR)/BR mice (25–30 g; Charles River Breeding Laboratories, Bloomington, MA) were maintained on a 12-h light/dark cycle with food and water available ad libitum. Mice were housed in groups of five until testing. [¹²⁵I]NaI (1680 Ci/mmol) was purchased from NEN (Boston, MA). Morphine, morphine-6β-glucuronide (M6G), and [D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO) were generously provided by the Research Technologies Branch of the National Institute on Drug Abuse (Rockville, MD). MK801 was purchased from Research Biochemicals, Inc. (Natick, MA).

Systemic drugs were given subcutaneously in the midscapular region of the back. Intracerebroventricular and intrathecal injections were performed under light halothane anesthesia 15 min before...
testing, as previously reported (Kolesnikov et al., 1996). The i.c.v. injections were administered 2 mm caudal and 2 mm lateral to the bregma at a depth of 3 mm, whereas intrathecal injections were made by lumbar puncture. Drugs were given topically on the tail by immersion of the tail in dimethyl sulfoxide (DMSO) solutions containing the indicated drugs. The distal portion of the tail (3 cm) was immersed in DMSO solution for 1 min. Tail-flick latencies were then determined on the region of the tail immersed in the drug, unless otherwise stated. To ensure a local effect, testing was also done with a more proximal segment of tail not exposed to the drug solution.

Analgesia was assessed with the tail-flick assay, as previously reported (Kolesnikov et al., 1996). The tail was exposed to a focused beam of light, and the latency of exposure was determined. Baseline latencies ranged from 2.5 to 3.5 s. A maximum cutoff latency of 10 s was used to minimize tissue damage in analgesic animals. Testing was performed 30 min after systemic administration, 15 min after either i.c.v. or i.t. injections or immediately after termination of topical administration into the tail. Antinociception, or analgesia, was defined quantally as a tail-flick latency for an individual mouse that was at least twice its baseline latency. Group comparisons were performed with the Fisher exact test. ED$_{50}$ values were determined with the Bliss program, as previously reported (Pick et al., 1993). To ensure local action, in all studies, we examined a region of the tail that was immersed in DMSO and a more proximal segment that was not exposed. Tail-flick latencies from the unexposed portion of the tail were similar to baseline latencies. DMSO itself had no activity in this model. Testing regions of the tail that were exposed and not exposed to DMSO revealed no significant antinociceptive effect in either location.

$^{125}$I-labeled morphine and $^{125}$I-labeled DAMGO were synthesized at room temperature with the chloramine-T method with equimolar amounts of $^{125}$I-NaI and either morphine or DAMGO. The reaction terminated with sodium metabisulfite after 1 min, and the radiolabeled opioid was separated from unreacted $^{125}$I-NaI by a C18 reverse-phase SepPak (Chien et al., 1997). The radiolabeled compounds were not further separated from the nonionized precursors.

## Results

**Topical Morphine and DAMGO Analgesia.** Prior studies from our group demonstrated a potent local analgesic activity of morphine administered s.c. in the tail (Kolesnikov et al., 1996). Morphine also was a potent analgesic when applied topically. The analgesic response to a morphine solution (7.5 mM) progressively increased over time, going from only 25% after 30 s to 50% by 1 min and 80% after 2 min (data not shown). The onset of the response was rapid. Analgesia was detectable within 1 min, the shortest time tested, after removal of the tail from the opioid solution (Fig. 1A). However, the morphine response was relatively brief, typically lasting less than 30 min. With a fixed exposure time, morphine produced a dose-dependent effect (Fig. 1B and Table 1). Similar results were observed with DMSO solutions of the $\mu$-opioid peptide DAMGO, which was over 5-fold more potent (Fig. 1B and Table 1). In addition to its greater potency, DAMGO had a longer duration of action, lasting almost 1 h (Fig. 1A). Like morphine, peak DAMGO actions were seen immediately after removal from the DMSO solution. These analgesic responses were easily reversed by systemic naloxone (1 mg/kg s.c.), confirming the opioid selectivity of the response (Fig. 2A). Furthermore, no analgesic response was seen with these agents in the proximal portions of the tail not exposed to the opioid solutions.

To further confirm the selectivity of the method, we looked at the distribution of radioactivity after immersion into a solution containing either $^{125}$I-labeled morphine or $^{125}$I-labeled DAMGO (Table 2). The region of the tail exposed to the solution had high levels of radioactivity. A more proximal portion of the tail that was not directly exposed to the solution had levels of radioactivity less than 1% of those in the distal portion of the tail immersed in the solution. Furthermore, little radioactivity was seen in blood, brain, or spinal cord.

**Topical Morphine-6$\beta$-Glucuronide Analgesia.** M6G administered locally by s.c. injection in the tail was analgesic, but it had a ceiling effect of 30% with doses of 10 or 30 $\mu$g (data not shown). In the topical paradigm, M6G yielded a full analgesic response, with peak effect immediately after removal from the solution (Fig. 1A) and a potency similar to

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Topical opioid analgesia in the mouse. A, groups of mice received a 2-min topical exposure to morphine (15 mM; n = 20), DAMGO (2 mM; n = 10), or M6G (20 mM; n = 10) and were tested in the tail-flick assay. B, dose-response curves were generated for each of the designated compounds applied topically for 1 min, as described in Materials and Methods. Each dose of drug had at least 10 mice/group.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ED$_{50}$ (95% CL)</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>8.3 (4–13)</td>
<td>1</td>
</tr>
<tr>
<td>M6G</td>
<td>9.3 (7–14)</td>
<td>0.9</td>
</tr>
<tr>
<td>DAMGO</td>
<td>1.6 (1–2.5)</td>
<td>5</td>
</tr>
</tbody>
</table>

### Table 1

Analgesic activity of topical opioids in C57BL/6J mice

Analytic ED$_{50}$ values with 95% confidence limits (CL) were determined by using at least three doses of drug in groups of mice (n = 10–20/dose) in the tail-flick assay. All drugs were administered topically for 1 min, as described in Materials and Methods. The ratios of M6G and DAMGO were determined against morphine. Ranges are in parentheses.
that of morphine (Fig. 1B; Table 1). As with morphine, proximal tail segments did not display analgesia, and the M6G response was readily reversed by systemic naloxone (Fig. 2A). The duration of M6G action after topical administration was similar to that of DAMGO and longer than that of morphine. The M6G-selective antagonist 3-methoxynaltrexone (3MeONtx) (Brown et al., 1997) also significantly lowered the M6G response (Fig. 2B). In contrast, the same 3MeONtx dose was inactive against the analgesic actions of morphine or DAMGO. In addition to supporting the selectivity of 3MeONtx for the M6G receptors, these observations strongly supported the presence of functional peripheral M6G receptors.

**Peripheral/Central Synergy.** Prior work from our laboratory suggested a potent synergy between peripheral and central morphine systems. We also examined these interactions after topical administration. The actions of morphine rapidly dissipated, falling from 80% at 1 min to only 30% at 10 min. No analgesia was seen by 30 min. Minimally active doses of i.t. or s.c. morphine markedly potentiated the response of topical morphine (Fig. 3). This was most dramatic at time points beyond 30 min, when the topical response alone was completely lost. At these longer time points, the analgesic responses of the combinations were significantly greater than their additive effects (Fig. 3B).

We next looked at the effects of a fixed dose of topical morphine on the ED50 values of spinal and systemic morphine (Table 3). Topical morphine potentiated the analgesic potency of systemic morphine almost 7-fold, even though it had no activity alone at the time point examined (30 min).

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>125I-Labeled DAMGO</th>
<th>125I-Labeled Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>69 ± 18</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Brain</td>
<td>55 ± 26</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>71 ± 12</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Tail</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed</td>
<td>38,460 ± 3,455</td>
<td>45,280 ± 2,637</td>
</tr>
<tr>
<td>Unexposed</td>
<td>234 ± 51</td>
<td>157 ± 51</td>
</tr>
</tbody>
</table>

**Fig. 3.** Interactions between topical and either systemic or spinal morphine. A, groups of mice (n ≥ 10) received topical morphine (15 mM; 2 min) alone, or with spinal (100 ng, i.t.) or systemic (1 mg/kg s.c.) morphine. The spinal morphine dose alone had no observable action, and the systemic dose produced only a 10% response. At 30 min, when the response to topical drug alone was lost, the responses of the combinations were significantly greater: ○, topical alone; □, with systemic; △, with spinal; *p < .002; **p < .04. B, left, groups of mice (n ≥ 10) received topical morphine (15 mM; 2 min) alone, spinal morphine (100 ng, i.t.) alone, or both together. Testing was performed 10 min after drug administration. At this time point, topical morphine alone had a 30% response. The combined dosing was significantly more active than the sum of the two individual routes alone. Right, groups of mice (n ≥ 10) received topical morphine (15 mM) alone, systemic morphine (1 mg/kg s.c.) alone, or both together. Testing was performed 30 min after drug administration. At this time point, topical morphine alone had no observable response. The combined dosing was significantly more active than the sum of the two doses alone. *p < .01.

Topical morphine also enhanced the potency of i.t. morphine almost 12-fold. Thus, these results support the earlier suggestions of potentiation between peripheral and central morphine analgesic systems.
Peripheral Morphine Tolerance. Peripheral systems are important in the production of tolerance after systemic morphine (Kolesnikov et al., 1996). The tail-immersion approach enables repeated local administration of drug without tissue damage, facilitating the study of peripheral morphine tolerance. Daily topical morphine (15 mM) produced profound tolerance by the 3rd day (Fig. 4), shifting morphine’s ED\textsubscript{50} value >9-fold (Table 4). Topical tolerance developed more rapidly and to a greater extent than that seen with daily systemic drug, where 5 days of treatment only shifted the morphine dose-response curve ~2-fold.

Mice given morphine systemically showed significant tolerance to topical morphine and to DAMGO (Fig. 5). However, the analgesic activity of topical M6G in these mice remained unchanged, confirming the lack of cross-tolerance reported previously (Rossi et al., 1996).

Blockade of Peripheral Morphine Tolerance by N-Methyl-d-Aspartate (NMDA) Antagonists. The NMDA/nitric oxide cascade plays an important role in the production of morphine tolerance (Kolesnikov et al., 1993). Blockade of this system prevents the development of morphine tolerance without interfering with analgesia. The NMDA antagonist MK801 given systemically also prevented the development of tolerance to topical morphine (Fig. 6A). Topical MK801 also blocked morphine tolerance as effectively as systemic drug (Fig. 6A), but i.t. MK801 was ineffective. Topical MK801 actions were dose dependent, with 0.3 mM effectively blocking tolerance (Fig. 6B).

Furthermore, topical MK801 could reverse preestablished tolerance (Fig. 6C). After treating mice with topical morphine alone for 3 days, the analgesic response was eliminated. Adding MK801 to the treatment regimen restored analgesic sensitivity over the next 2 days despite the continued administration of morphine. The higher MK801 dose was slightly more effective than the lower one. The slow rate reversal with no effect after the first dose argues strongly against a simple potentiation of morphine potency.

Fig. 4. Tolerance to systemic and topical morphine. Groups of mice (n = 10) received morphine either systemically (5 mg/kg s.c.) or topically (15 mM; 1 min). DMSO alone had no observable effect on days 1, 2, or 3. On day 3, the response in the systemic group was significantly greater than the topical group. *p < .003.

Discussion

Peripheral opioid actions are becoming increasingly important in the understanding of opioid actions, as demonstrated by the role of peripheral and central synergy in the actions of systemic morphine (Kolesnikov et al., 1996). Furthermore, peripheral sites of action play a major role in the development of tolerance to systemic drug. Exploring peripheral mechanisms is not simple. Earlier studies used local injections into the tail to examine peripheral mechanisms. Although useful, this approach has several disadvantages, particularly when looking at repeated dosing. To avoid this problem, we developed a topical approach that is generally applicable to both alkaloids and peptides. The tail-immersion technique has several advantages. Foremost is the ability to repeatedly treat the mice without tissue damage secondary to injections. The paradigm was selective for local mechanisms. Testing proximal regions of the tail failed to reveal any analgesic response, confirming the distribution studies with \textsuperscript{125}I-labeled opioid, which documented the localization...
of the radiolabel only to the regions immersed in the drug solution and the absence of any detectable uptake into the blood or central nervous system. Equally important, DMSO alone had no effect in the tail-flick assays. Presumably, the activity of this approach is not limited to DMSO, and other solvents or topical creams could be used. We had not anticipated that topical solutions of peptides would be active, but several different \( \mu \) - and \( \delta \)-peptides are effective in this paradigm. Clearly, topical approaches open new possibilities clinically for these peptides, which are not very effective systemically. Thus, the topical approach provides is useful in the examination of peripheral opioid mechanisms and potential utility in pain management.

Peripherally, all the opioids tested were effective analgesics. Of the three, DAMGO was the most active. The similar potencies of morphine and M6G peripherally contrast with their central actions, where M6G is approximately 100-fold more active than morphine. In all cases, the proximal segments of the tail that were not exposed to the opioid solution were not analgesic, confirming the peripheral site of action for the sites immersed in the opioid solution. The responses were readily antagonized by naloxone. Centrally, 3MeONtx selectively reverses M6G analgesia without interfering with morphine analgesia, consistent with a different receptor mechanism of action (Brown et al., 1997). 3MeONtx also reversed peripheral M6G analgesia without affecting either DAMGO or morphine action. Thus, peripheral M6G analgesia showed the same antagonist selectivity as seen centrally. Prior studies had documented synergy between peripheral and central morphine actions. The current study confirms the earlier observations. Combining topical morphine with morphine given either systemically or spinally revealed marked potentiation of the responses beyond those expected for simple additive interactions. Thus, if topical opioids were

Fig. 6. Effects of MK801 on topical morphine tolerance. A, groups of mice (\( n \geq 10 \)) received topical morphine (15 mM; 1 min) alone or with MK801 given topically (3 mM), systemically (0.1 mg/kg s.c.), or intrathecally (1 \( \mu \)g, i.t.). After 3 days, the response to morphine alone was lost (\( p < .01 \)), as was the response to morphine with intrathecal MK801 (\( p < .01 \)). The combination of morphine with either systemic or topical MK801 remained essentially unchanged for 5 days. \( \bullet \), morphine alone; \( \Delta \), with topical MK801; dashed line, with systemic MK801. B, groups of mice (\( n \geq 10 \)) received topical morphine (15 mM; 1 min) alone, topical MK801 alone (3 mM), or topical morphine (15 mM) with topical MK801 at the indicated concentration (0.15, 0.3, or 3 mM). After 3 days, the response to morphine alone was lost (\( p < .01 \)). The two higher MK801 doses prevented the loss of responsiveness (\( p < .01 \)), whereas the lowest doses gave an intermediate response. \( \bullet \), morphine alone; \( \Delta \), with MK801 (3 mM); \( \triangle \), with MK801 (0.3 mM); \( \mathcal{V} \), with MK801 (0.15 mM); \( \checkmark \), MK801 alone (3 mM). C, groups of mice (\( n \geq 10 \)) received topical morphine (15 mM; 1 min) alone for 3 days. Starting on day 4, they received topical morphine with topical MK801 at either 0.3 or 3 mM. Coadministration of topical MK801 with topical morphine reversed the previously established tolerance (\( p < .01 \)).
to be tried clinically, the results suggest they would be more effective in combination with systemic dosing. By lowering the necessary doses of systemic drug, topical opioids might greatly diminish the side effects currently associated with opioid analogues.

Chronic dosing with systemic morphine treatment leads to tolerance. Localizing the site of morphine tolerance has been difficult. Mice tolerant to systemic morphine show normal sensitivities to morphine given either spinally or supraspinally (Roerig et al., 1984) but not peripherally (Kolesnikov et al., 1996). Indeed, the 19-fold shift in local morphine dose-response curves far exceeds the shift after systemic administration. Our current studies support a role for peripheral sites in morphine tolerance. Chronic topical morphine produced tolerance rapidly, decreasing the response to undetectable levels by 3 days, corresponding to a >9-fold shift in the dose-response curve. Chronic dosing with DMSO alone had no effect. The rate of development of tolerance to equianalgesic doses of systemic drug was slower and less, shifting the dose-response curve only 2-fold after 5 days. Mice tolerant to peripheral morphine were cross-tolerant to DAMGO but not to M6G. This lack of cross-tolerance is consistent with the selective reversible of M6G analgesia by 3MeONtx and with a unique receptor mechanism of M6G action.

NMDA-receptor antagonists or nitric oxide synths inhibitors prevent the production of morphine tolerance (Ben-Eliyahu et al., 1992; Gutstein and Trujillo, 1993; Kolesnikov et al., 1993; Trujillo and Akil, 1994). In view of the importance of peripheral opioid mechanisms in tolerance in these paradigms, we looked at the role of peripheral NMDA antagonists. Topical morphine tolerance was effectively blocked by MK801 given systemically or topically but not spinally. Systemic MK801 would be expected to have access throughout the animal, including peripheral sites, whereas the i.t. drug would be restricted to central sites. Thus, only treatments with access to peripheral sites were active in this model, implying that peripheral NMDA receptors are responsible for mediating topical morphine tolerance. Recent evidence supports the presence of excitatory amino acid receptors on peripheral cutaneous axons (Carlton et al., 1995; Zhou et al., 1996; Davidson et al., 1997). Additional studies are needed to verify the site of action. However, the activity of topical NMDA antagonists opens many clinical possibilities in pain management. Many of the current NMDA-receptor antagonists open the way for many highly selective agents acting through non-μ-opioid-receptor mechanisms. Finally, the ability to block topical tolerance with peripherally acting NMDA antagonists is another exciting possibility in the clinical treatment of pain. It will be interesting to determine whether similar analgesic mechanisms can be exploited in humans.

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References
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