Subarachnoid Morphine Reduces Stimulation-induced but Not Basal Expression of Preproenkephalin in Rat Spinal Cord

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Background: To evaluate directly the possibility that the potent exogenous opioid analgesic morphine may alter neuronal expression of opioid peptide genes, we assessed the effect of subarachnoid morphine on basal and noxious stimulation-induced expression of preproenkephalin in spinal cord neurons.

Methods: Twenty male Sprague-Dawley rats were prepared 48 h in advance with lumbar subarachnoid catheters. In the first phase, basal expression was evaluated in rats that received morphine 10 µg or saline intrathecally (n = 5 per group). Subsequently, the experiment was repeated (n = 5 per group), except that 10 min after morphine or saline administration rats received a hindpaw footpad injection of 50 µl 5% formalin. Rats were killed during pentobarbital anesthesia 2 h later, and messenger RNA transcribed from preproenkephalin was measured in lumbar spinal cord with quantitative in situ hybridization with a complementary sulfur 35-labeled oligonucleotide probe and emulsion autoradiography.

Results: In control (nonstimulated) rats, 20% of the neurons in laminae I–II and 10% of those in laminae III–IV expressed preproenkephalin. Injection of formalin increased the fraction of positive neurons by 34% (P < 0.05) and 20% (P < 0.05) in laminae I–II and V–VI, respectively, but had no effect on expression in laminae III–IV. Subarachnoid morphine did not alter basal expression of preproenkephalin but markedly attenuated the noxious stimulation–induced increase in laminae I–II (P < 0.01) and V–VI (P < 0.05) by preventing the stimulation-evoked recruitment of preproenkephalin-expressing neurons that otherwise would have occurred.


Tissue damage in the periphery leads to numerous changes in central neural function that influence the subsequent pain experience.1 Central release of excitatory neurotransmitters such as the amino acids glutamate and aspartate1,2 and the tachykinin substance P3,4 from primary afferent terminals serve to facilitate or sustain pain, whereas release of neurochemical mediators with inhibitory functions may modulate nociceptive transmission.5–7 For instance, the opioid peptides leu- and metenkephalin are released from local and/or descending inhibitory neurons after noxious peripheral stimulation4–7 and there is a rapid corresponding increase in neural expression of preproenkephalin,8–11 the gene encoding the message for these neurotransmitters. Preproenkephalin messenger RNA (mRNA) levels increase 20–40% in appropriate regions of the brainstem after intense electrical stimulation of the trigeminal nerve10,11 and increase approximately 50% in the spinal cord dorsal horn after injection of a noxious substance such as formalin into the paw of a rat.8,9 These increases in preproenkephalin mRNA occur within 1–2 h of the onset of stimulation, are selective for neurons known to be intimately involved in modulation of nociceptive inputs (i.e., those in laminae I–II and V–VI), and are closely linked to activity in small-diameter primary afferent fibers.8–11 In conjunc-
tion with the recognized analgesic properties of both endogenous and exogenous opioid receptor agonists, these observations suggest that these noxious stimulation–induced increases in opioid peptide release and gene expression represent an important, although functionally still somewhat poorly defined, physiologic response to pain.

Exogenous opioid analgesics such as morphine mimic the action of endogenous opioid neurotransmitters at receptors located both presynaptically on the central terminals of primary afferent fibers and postsynaptically on thalamic projection neurons and in this way alter normal neural responses to noxious stimulation. Morphine and other μ-opioid receptor agonists, for example, block noxious stimulation–evoked release of excitatory neurotransmitters and modulators from nociceptive primary afferent fibers and thereby limit the central, transsynaptic propagation of the peripheral noxious stimulus. Because noxious stimulation increases expression of preproenkephalin and morphine interferes with stimulatory neurochemical events necessary for the transmission and maintenance of pain, we speculated that administration of morphine would prevent the painful stimulation-induced increase in neuronal expression of preproenkephalin that would otherwise occur physiologically. In fact, morphine does acutely attenuate stimulation-induced expression of a nuclear protein, fos, that is coexpressed by most opioid neurons and thought to play an important role in regulating expression of preproenkephalin. In addition, because morphine reduces basal expression of preproenkephalin, albeit after prolonged administration and in brain regions not directly involved in nociception, we also anticipated that it might acutely reduce basal expression of preproenkephalin in the spinal cord. Accordingly, this study was undertaken to investigate the effect of acute subarachnoid administration of morphine on both basal and stimulation-induced expression of preproenkephalin in neurons of the spinal cord dorsal horn.

Materials and Methods

Experiments were performed with approval of the institutional Subcommittee on Animal Care in 27 male 300–325-g Sprague-Dawley rats. Preproenkephalin expression was evaluated with quantitative in situ hybridization. Conceptually, this technique is analogous to immunohistochemistry or receptor autoradiography except that a specific mRNA, instead of a peptide or receptor, is identified as the “reporter” molecule by hybridization with a radiolabeled DNA or RNA oligonucleotide probe complementary to the mRNA of interest. In these experiments, mRNA transcribed from preproenkephalin was identified by hybridization with a complementary sulfur 35–labeled DNA probe.

Animals were prepared approximately 48 h in advance with a lumbar subarachnoid catheter as described previously. During 1% halothane–70% nitrous oxide anesthesia, a 15-cm length of polyethylene tubing (PE 10) was advanced 8 cm caudally into the subarachnoid space from a slit in the cisternal membrane. The catheter was then brought out the back of the animal’s neck and secured in place. Rats were evaluated clinically for ability to walk, groom, and stand on their hindlimbs on awakening and again just before the experiment. Catheter position and function were also confirmed the day before experimentation by testing for hindlimb sensory and motor blockade after a single intrathecal dose of lidocaine (10 μl of a 2% solution). Only neurologically normal animals with functioning catheters were used for the experiments.

To assess the effect of morphine on basal expression of preproenkephalin, rats received either morphine, 10 μg (n = 5), or an equivalent volume of saline (n = 5) intrathecally. Animals used in the stimulation experiments (n = 5 per group) were treated identically except that 10 min after subarachnoid morphine or saline were administered they received an injection with a 25 gauge needle of 50 μl of 5% formalin into the plantar surface of a hindpaw footpad. The dosage of morphine was selected on the basis of previous studies in rats and preliminary experiments in our laboratory which demonstrated that this dose produced greater than 2 h of near-maximal analgesia on the tail-flick test. Morphine was prepared in preservative-free saline and administered in a 10 μl volume followed by an additional 15 μl of saline to flush the catheter. Control animals received 25 μl of saline intrathecally. Apart from handling for administration of morphine or footpad injection, rats were left undisturbed in their cages until killing 2 h after morphine treatment. Analgesia was assessed with the tail-flick test before drug administration and again just before killing. For this purpose, the tail was placed over a slit 1.5 cm from a focused 150-W projection bulb. The end-point of the test was removal of the tail; a 6-s cut off was used to avoid thermal damage.
At the time of killing, rats were anesthetized with intraperitoneal pentobarbital and perfusion-fixed through the left ventricle with phosphate-buffered saline and a solution of 0.5% depolymerized paraformaldehyde, 1% glutaraldehyde, 75 mEq/l sodium phosphate, 37.5 mEq/l sodium phosphate (pH 7.4), and 10 mM sodium metaperiodate to approximate 1 ml/mg body weight as described previously.10,11,20 The lumbar spinal cord was removed, postfixed in 0.5% depolymerized paraformaldehyde, 1% glutaraldehyde, 75 mEq/l lysine, 37.5 mEq/l sodium phosphate (pH 7.4), and 10 mM sodium metaperiodate for 90 min at 4°C; soaked for 30 min in 15% sucrose in phosphate buffer; and rapidly frozen and stored at −70°C. Tissue sections 10 µm thick were cut in a cryostat and thaw-mounted onto slides coated with Denhardt’s solution and pretreated with acetylation. Sections were then treated with 0.2 M hydrochloric acid and proteinase K, dehydrated in ethanol, and air dried at room temperature. Serial sections were hybridized with a 48-base, 35S-labeled oligonucleotide probe complementary to mRNA transcribed from the preproenkephalin gene.10,11,20 Probe was synthesized chemically using an Applied Biosystems oligonucleotide synthesizer. Antisense 12-base DNA primers complementary to regions beginning three to five bases from the 3' end of the template strand mRNA were synthesized and allowed to hybridize to each other. The antisense strand was extended using Klenow fragment of DNA polymerase I, 35S-labeled deoxycytidine triphosphate and deoxyadenosine triphosphate (Amersham, Arlington Heights, IL) and unlabeled deoxyguanosine triphosphate and deoxythymidine triphosphate. Radiolabeled probe was separated from template by denaturing polyacrylamide gel electrophoresis. This complementary DNA probe is highly specific for rat preproenkephalin mRNA, shows no cross-reactivity with other mRNA species, and as demonstrated in our laboratories previously,10,11,20 the tissue signal is eliminated by digestion of mRNA with ribonuclease and by application of excess nonradiolabeled oligonucleotide in competition experiments.

Each tissue-containing slide was treated with 250 × 10^3 counts/min of 35S-labeled preproenkephalin complementary DNA in hybridization buffer and then covered with a coverslip. Hybridization was allowed to occur for 24 h; slides were then washed to remove free probe, dipped in liquid photographic emulsion (Kodak NTB2, Eastman Kodak, Rochester, NY), and exposed for 7 days. Emulsion coated slides were developed according to standard procedures and tissue sections were stained with toluidine blue to permit identification of spinal laminae and cell morphologic features. The amount of hybridization was quantified microscopically10,11,20 in individual neurons in laminae I–II and III–IV. In formalin injected rats, expression was also evaluated in laminae V–VI because, like laminae I–II, this area is known to receive noxious somatosensory inputs.24 After a neuron was identified, the plane of focus was shifted from the tissue to the emulsion and autoradiographic grains lying in a 10 × 10–µm zone overlying the neuron were counted using a microscope equipped with a calibrated eyepiece and 100× objective magnification. This process was repeated for every identifiable neuron lying within the 10 × 10–µm zone and throughout each of the laminae in at least four sections. The background grain density was obtained for each section from a 10 × 10–µm area of adjacent white matter. An abbreviated, previously described version of this procedure was used for the seven rats included in the naloxone reversal subset of the study (i.e., only positive neurons were counted). Numerical values for the mean grain density per neuron, mean grain density per positive neuron (defined as a neuron with greater than 3× background grains), and fraction of positively hybridizing neurons in the nonstimulated morphine-treated and the corresponding control group were compared using a group t test. In stimulated animals, side-to-side differences were analyzed with a paired t test and percentage side-to-side differences between groups compared with a group t test.

Results
As anticipated, subarachnoid morphine maximally prolonged the latency of the tail-flick response (100% maximum percentage effect; P < 0.01). Grain counting was performed on more than 1,100, 700, and 1,000 neurons per group in laminae I–II, III–IV, and V–VI, respectively (differences reflect regional variation in neuronal density). In control (nonstimulated) animals, only 20% and 10% of neurons in laminae I–II and III–IV, respectively, expressed preproenkephalin (table 1). Subcutaneous injection of formalin in control rats increased the fraction of positive neurons 34% (P < 0.05) and 20% (P < 0.05) in laminae I–II and V–VI, respectively, compared to the contralateral, nonstimulated side (table 2; fig. 1) and increased the mean grain count per neuron to a similar extent. However, in laminae III–IV, a region that does not receive noxious somato-
SPINAL MORPHINE AND PREPROENKEPHALIN EXPRESSION

Table 1. Effect of Morphine on Constitutive Expression of Preproenkephalin in Laminae I–II and III–IV Neurons of the L5 Segment of Rat Spinal Cord

<table>
<thead>
<tr>
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<th>Saline (n = 5)</th>
<th>Morphine (n = 5)</th>
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<tbody>
<tr>
<td>Laminae I–II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grains/neuron</td>
<td>3 ± 0.3</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>Grains/positive neuron</td>
<td>13 ± 0.6</td>
<td>13 ± 1.0</td>
</tr>
<tr>
<td>Fraction positives (%)</td>
<td>20 ± 1.7</td>
<td>22 ± 1.4</td>
</tr>
<tr>
<td>Laminae III–IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grains/neuron</td>
<td>2 ± 0.3</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>Grains/positive neuron</td>
<td>14 ± 1.6</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>Fraction positives (%)</td>
<td>10 ± 1.4</td>
<td>8 ± 1.1</td>
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</tbody>
</table>

Values are mean ± SEM for the number of animals in parentheses and at least 100 neurons per animal per region. P > 0.1 for all comparisons.

sensory inputs, stimulation did not influence expression of preproenkephalin (table 2).

In nonstimulated rats, subarachnoid morphine had no effect on the mean grain count per neuron or the mean grain count per positive neuron (both are indices of the intensity of preproenkephalin expression) and did not change the fraction of positive neurons (table 1). In contrast, morphine administered before injection of formalin markedly attenuated the stimulation-induced increase in the fraction of positive neurons in laminae I–II (10% increase vs. 34% in the control group; P < 0.01) and V–VI (1% increase vs. 20% in the control group; P < 0.05) (table 2 and fig. 1). Morphine had no effect on stimulation-evoked increases in the mean grain count per neuron or the mean grain count per positive neuron in any laminae, however.

Discussion

These data demonstrate that a noxious peripheral stimulus quickly induces neurons in pain-sensitive laminae of the spinal cord dorsal horn (i.e., laminae I–II and V) to express preproenkephalin at higher than normal levels. This confirms previous studies and additionally shows that the principle mechanism of the formalin-induced increase in preproenkephalin expression is recruitment of a subpopulation of previously nonexpressing neurons to express the gene (as indicated by a 20–34% increase in the fraction of expressers). Subarachnoid analgesia with morphine before footpad injection markedly attenuated this stimulation-evoked response primarily by preventing the recruitment of preproenkephalin-expressing neurons that otherwise would have occurred. Because subarachnoid morphine did not change basal expression of preproenkephalin (i.e., did not alter expression in nonstimulated rats), a direct action of morphine on preproenkephalin-expressing cells can not explain its effects on stimulated expression of this gene. In fact, the effect of morphine was selective even in stimulated animals for those laminae that otherwise would have expressed preproenkephalin at higher levels due to formalin-induced pain (i.e., I–II and V–VI, but not laminae III–IV). Hence, we conclude that morphine acts indirectly to reduce stimulation-evoked expression of preproenkephalin by blocking noxious excitatory inputs into opioid neurons. This mechanism is consistent with the acknowledged ability of morphine and other µ opioid receptor agonists to inhibit, via a presynaptic action on the central terminals of primary afferent fibers, stimulation-evoked release of primary afferent neurotransmitters and does not preclude the possibility that morphine reduces the sensitivity of second-order neurons to noxious inputs. Moreover, because opioid neurons lack reuptake mechanisms for intact peptide and must synthesize each molecule de novo by an mRNA mediated process, the data are also concordant with evidence that morphine suppresses noxious stimulus-evoked release of enkephalin from the spinal cord. Accordingly, we predict that the ability

Table 2. Percentage Change in Preproenkephalin Expression Ipsilateral to Noxious Stimulation versus the Contralateral Side in Control and Morphine-treated Rats

<table>
<thead>
<tr>
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<th>Stimulation-Induced Change (%)</th>
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<tbody>
<tr>
<td></td>
<td>Saline (n = 5)</td>
</tr>
<tr>
<td>Laminae I–II</td>
<td></td>
</tr>
<tr>
<td>Grains/neuron</td>
<td>34 ± 13*</td>
</tr>
<tr>
<td>Grains/positive neuron</td>
<td>1 ± 11</td>
</tr>
<tr>
<td>Fraction positives (%)</td>
<td>34 ± 3*</td>
</tr>
<tr>
<td>Laminae III–IV</td>
<td></td>
</tr>
<tr>
<td>Grains/neuron</td>
<td>11 ± 14</td>
</tr>
<tr>
<td>Grains/positive neuron</td>
<td>1 ± 7</td>
</tr>
<tr>
<td>Fraction positives (%)</td>
<td>9 ± 13</td>
</tr>
<tr>
<td>Laminae V–VI</td>
<td></td>
</tr>
<tr>
<td>Grains/neuron</td>
<td>23 ± 8*</td>
</tr>
<tr>
<td>Grains/positive neuron</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>Fraction positives</td>
<td>20 ± 8*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for the number of animals in parentheses.

* P < 0.05 for side to side comparisons by paired t test.
† P < 0.01 versus control group by pooled t test.
‡ P < 0.05 versus control group by pooled t test.

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of an anesthetic/analgesic agent to attenuate noxious stimulation–evoked expression of preproenkephalin will depend in part on the degree to which it blocks noxious afferent input to the spinal cord. In this regard, because electrophysiologic studies demonstrate that posttreatment with morphine is several orders of magnitude less effective than pretreatment in preventing noxious stimulation–evoked discharges of spinal nociceptive neurons, even morphine may have a less prominent effect on preproenkephalin expression if it is administered after noxious stimulation.

We focused on preproenkephalin because its transcription products (i.e., mRNA) encode two of the most abundant analgesic peptide neurotransmitters in the central nervous system, namely, leu- and methenkephalin. Moreover, several lines of evidence, including the fact that stimulation-evoked increases in expression of preproenkephalin occur selectively in segmental neurons responsive to high intensity stimuli and can be modulated by an exogenous analgesic such as morphine (table 2; fig. 1), suggest a role for this gene and its products in nociceptive processes. Preprodynorphin has also been implicated in nociception, however. Although the percentage increase in expression of preprodynorphin after noxious stimulation is larger than that of preproenkephalin, the increase reflects recruitment of relatively few neurons because preprodynorphin mRNA is barely detectable in the spinal cord under basal conditions. In contrast, based on our empirically derived estimates of the total number of preproenkephalin-expressing neurons per side of laminae I–II of the spinal L5 segment of the rat (about 7,500 neurons), we calculate that formalin stimulation recruited within 2 h approximately 2,000 additional neurons to express preproenkephalin.

Expression of preproenkephalin is a dynamic and highly regulated process that is incompletely understood. It is apparent from this study and others, however, that sensory input has a powerful influence. Peripheral stimulation produced either electrically or by injection of inflammatory agents such as formalin augments expression of preproenkephalin in segmental pain-sensitive neurons of the spinal cord or brainstem and increases synthesis of its neurotransmitter protein products, leu- and methenkephalin. Conversely, nerve transection or selective destruction of primary afferent fibers reduces preproenkephalin transcripts as much as 30–45%. Accordingly, taken together with the fact that morphine inhibits noxious somatosensory inputs, it is likely that selective pharmacologic deafferentation accounts for morphine’s action on stimulation-induced expression of preproenkephalin. On this basis, no effect of morphine on basal expression of preproenkephalin would be expected because there is little or no tonic noxious input to the spinal cord under normal circumstances. Furthermore, although morphine is an enkephalin agonist and there is precedent for negative feedback inhibition of the parent neuron by its natural transmitter or an analog, failure of morphine to influence basal expression of preproenkephalin indicates it does not acutely control spinal opioid-producing neurons in this manner. Chronically administered morphine may act differently, however, because it reduces preproenkephalin mRNA levels in the caudate and paraventricular nuclei of the brain. In this context, it must be emphasized that a 2-h experimental interval is sufficient for a decrease in preproenkephalin expression to be manifest; preproenkephalin mRNA decreases more than 40% from peak stimulated levels within just 1–2 h of discontinuing stimulation and decreases 25–30% below baseline within 2 h of a single intravenous bolus of ketamine or continuous anesthesia with pentobarbital or halothane.

**Fig. 1.** Percentage change in the fraction of neurons expressing preproenkephalin ipsilateral to noxious stimulation versus the contralateral side. Data are means and standard errors for five animals per group. *P < 0.05 for side-to-side comparisons. †P < 0.01 versus control group. ‡P < 0.05 versus control group.
Changes in afferent input are linked to subsequent alterations in neuronal expression of preproenkephalin by at least several intracellular signals.29 Nuclear proteins derived from a large family of genes called immediate-early genes are thought to be particularly important in this regard.16,17,37 These genes, most notably c-fos and jun-B, are expressed at low levels during resting conditions in brain and spinal cord neurons but undergo rapid and marked induction with increases in afferent activity.8,15,17,38 The protein products of these genes are capable of binding as homo- and heterodimers to specific regulatory sequences in the promoter region of preproenkephalin and regulating transcriptional activity of the gene.16,17,37 It is interesting, therefore, that morphine produces a dose-dependent decrease in noxious stimulation–evoked expression of c-fos protein in spinal cord neurons.13,14 In fact, the effect of morphine on spinal cord expression of c-fos parallels its effect on preproenkephalin in two respects: suppression of stimulation-induced expression of both genes is greater in laminae V–VI than I–II and expression of fos protein and preproenkephalin mRNA in superficial dorsal horn neurons persists despite maximal behavioral analgesia.13 Nonetheless, it is premature to conclude that morphine prevents stimulation-evoked expression of preproenkephalin by interfering with c-fos–mediated regulation of the gene because recent experiments challenge the view that c-fos is critical to regulation of preproenkephalin.30

Inasmuch as increased expression of preproenkephalin in the spinal cord dorsal horn is a physiologic, presumably adaptive, response to noxious stimulation, it may seem paradoxical that exogenous analgesia with morphine blunts the response. This in no way suggests that morphine is antinociceptive, however. To the contrary, as noted previously, the fact that morphine blocks noxious stimulation–induced release of primary afferent neurotransmitters1,2,12 probably accounts for both reduced expression of preproenkephalin after noxious stimulation and, as documented in numerous laboratory and clinical studies, its long term beneficial effects on pain behavior and analgesic requirements.1 Nevertheless, it may be possible to provide exogenous analgesia without reducing preproenkephalin expression and opioid neuron activity. It has recently been postulated,39 for example, that subarachnoid co-administration of nanomolar concentrations of morphine and the algesic peptide substance P augments release of spinal opioids. Thus, it is conceivable that novel analgesic regimens may actually enhance the activity and contribution of endogenous opioid neurons to the analgesic state.

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References

15. Noguchi K, Dubner R, Ruda MA: Preproenkephalin mRNA in spinal cord dorsal horn neurons is induced by peripheral inflammation and is co-localized with Fos and Fos-related protein. Neuroscience 46:561–570, 1992