σ₁ Receptor Modulation of Opioid Analgesia in the Mouse

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ABSTRACT
Opioid analgesia is influenced by many factors, including the σ₁ receptor system. Current studies show the importance of supraspinal mechanisms in these σ₁ actions. Given supraspinally, the σ₁ receptor agonist (+)pentazocine diminished systemic μ, δ, κ₁, and κ₂ opioid analgesia in CD-1 mice. There was a trend for the κ drugs to be more sensitive to the fixed dose of (+)pentazocine, although the differences did not achieve statistical significance. In contrast to its actions supraspinally, (+)pentazocine was without effect against morphine when both were given spinally. These findings are consistent with a supraspinal site of anti-opioid action of (+)pentazocine. Down-regulating supraspinal σ₁ binding sites using an antisense approach potentiated μ, δ, κ₁, and κ₂ analgesia in CD-1 mice. Although equally responsive to μ drugs, BALB-c mice are far less sensitive to κ analgesics than CD-1 mice. Earlier studies reported that these different responses to κ drugs between CD-1 and BALB-c were eliminated by the concurrent administration of haloperidol, a σ₁ antagonist. Antisense treatment of BALB-c mice markedly enhanced the response to κ drugs, as well as morphine. This enhanced response following antisense treatment was similar to that seen with haloperidol. These observations confirm the importance of σ₁ receptors as a modulatory system influencing the analgesic activity of opioid drugs.

Originally proposed from studies with the benzomorphphan (±)-N-allyl-normetazocine [(±)SKF-10047] (Martin et al., 1976), σ receptors are now defined as nonopioid, non-phenylcycloidal, haloperidol-sensitive, naloxone-inaccessible, (+)benzomorphine-selective binding sites (Quirion et al., 1992). Two subtypes of σ receptors have been proposed based upon their binding selectivity profiles (Bowen et al., 1993). σ receptors are conserved across species (Weissman et al., 1988; Su and Wu, 1990; Walker et al., 1992) and are present in almost all tissues, with very high expression in the central nervous system, immune system, and liver (Gundlach et al., 1986; Ryan-Moro et al., 1996).

The σ₁ receptor was first cloned from guinea pig liver (Hanner et al., 1996), followed by human (Kekuda et al., 1996), mouse (Pan et al., 1998), and rat clones (Seth et al., 1997, 1998; Mei and Pasternak, 2001). Structurally, σ₁ receptors show no homology with any traditional receptor family. Even with the new information available from cloning, many questions regarding the functional significance of σ₁ receptors remain, although recent work has reported an association with ankyrin (Hayashi and Su, 2001). Functionally, σ₁ receptors comprise an anti-opioid system and evidence suggests that some of the differences in sensitivity among strains of mice can be attributed to differing tonic levels of σ₁ receptor activity (Chien and Pasternak, 1993, 1994, 1995a,b; King et al., 1997). Haloperidol, a presumed σ₁ antagonist, potentiates systemic opioid analgesia, whereas the σ₁ ligand (+)pentazocine diminishes it. These modulatory functions apply to μ, δ, κ₁, and κ₂ analgesics. However, haloperidol is not selective for σ₁ receptors, displaying equally high affinity for D₂ dopamine receptors. Uncertainty regarding its actions is further raised by the observation that dopamine D₂ receptors also influence opioid actions (King et al., 2001). The present study compares the actions of (+)pentazocine and haloperidol with the effects of down-regulation of σ₁ receptors by an antisense approach to further define the role of σ₁ systems in opioid analgesia.

Materials and Methods
(+)-Pentazocine, morphine sulfate, trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate hydrate, (U50,488H), nalorphine, nalbuphine, and [d-Pen²,d-Pen⁵]enkephalin (DPDPE) were gifts from the National Institute on Drug Abuse Research Technology Branch (Rockville, MD). Halothane was purchased from Halocarcon Laboratories (Hackensack, NJ). Haloperidol, ketamine, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Naloxone benzoylhydrozone was purchased from Sigma-Aldrich (St. Louis, MO). Naloxone benzoylhydrozone is a specific antagonist of μ and δ opioid receptors.

ABBREVIATIONS: (±)SKF-10097, (±)-N-allyl-normetazocine, DPDPE, [d-Pen²,d-Pen⁵]enkephalin; NalBzoH, naloxone benzoylhydrozone; AS1, antisense treatment; bp, base pair; U50,488H, trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate hydrate.
(NalBzoH) was synthesized, as previously reported (Luke et al., 1988). All the drugs used in the in vivo studies were dissolved in saline, and doses were reported as the free base.

Male CD-1 (Crl:CD-1(ICR)BR) and BALB/c (BALB/cAnNCrlBR) mice (25–30 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and maintained on a 12-h light/dark cycle with rodent chow and water available ad libitum. All animal studies were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center and adhere to National Institutes of Health guidelines. Spinal (i.t.) and supraspinal (i.c.v.) injections were made under halothane anesthesia as previously described (Haley and McCormick, 1957; Hylden and Wilcox, 1980). The anesthetic did not affect analgesia measurements 15 min later.

All [3H] (+)pentazocine binding assays were performed as previously reported (Mei and Pasternak, 2001). In brief, membrane homogenates (100 μg of protein/ml) were incubated at 37°C for 3 h in potassium phosphate buffer (10 mM, pH 7.2) with [3H] (+)pentazocine (2 nM) and then filtered over glass fiber filters (Schleicher & Schuell, Keene, NH). All experiments were performed in triplicate and replicated at least three times. Only specific binding was reported, defined as the difference between total and nonspecific binding, as defined by that remaining in the presence of haloperidol (1 μM).

Analgesia was determined using the radiant heat tail-flick technique (D’Amour and Smith, 1941; Gistrak et al., 1990; Paul et al., 1990, 1991; Chien and Pasternak, 1994). Baseline latencies were determined before each experimental treatment for all animals as the mean of two trials and generally ranged between 2 and 3 s. Tail-flick latencies were measured again 15 min after either i.c.v. and i.t. injections and 30 min following s.c. injections. These times correspond to peak effects of the drugs (Paul et al., 1989; D. Paul and G. W. Pasternak, unpublished observations). A maximal latency of 10 s was used to minimize tissue damage. Analgesia was defined quantally as a doubling or greater of the baseline latency for each drug. A maximal latency of 20 s was used to minimize tissue damage. Analgesia was defined as the difference between total and nonspecific binding, as defined by that remaining in the presence of haloperidol (1 μM).

Results

Anti-Opioid Properties of α1 Receptors in the Central Nervous System. When administrated systemically (s.c.), (+)pentazocine antagonized both systemic and central morphine analgesia (Chien and Pasternak, 1994, 1995a,b). To determine the site of action of (+)pentazocine, we examined its effects when given centrally. (+)Pentazocine alone had no effect on tail-flick latencies when given intracerebroventricularly (data not shown). Supraspinal (+)pentazocine significantly reduced the analgesic actions of systemic and supraspinal morphine (Figs. 1 and 2). Spinal morphine analgesia was unaffected by (+)pentazocine given either i.c.v. or i.t.

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The actions of (+)pentazocine were not limited to the μ analgesic morphine. Supraspinal (+)pentazocine significantly diminished the analgesic activity of a wide range of systemically administered analgesics, including the κα agent U50,488H and the κα, κβ, κγ analgesic NalBzoH (Fig. 1). Although nalorphine is a partial agonist at μ receptors, its predominant analgesic actions are mediated through κα receptors (Paul et al., 1991). Nalbuphine, on the other hand, elicits its analgesic actions through a combination of κα and κβ receptors (Pick et al., 1992). As with the prototypical κα and κβ drugs, (+)pentazocine given supraspinally decreased the analgesic actions of both nalorphine and nalbuphine (Fig. 1).
Finally, supraspinal (+)-pentazocine significantly decreased the analgesic activity of supraspinal DPDPE and nalbuphine analgesia as effectively as supraspinal morphine (Fig. 2).

**Potentiation of Opioid Analgesia by σ₁ Receptor Antisense Treatment.** We next examined σ₁-opioid interactions at the molecular level by targeting the σ₁ receptors using an antisense approach. To validate the technique, we first confirmed the down-regulation of σ₁ receptor binding following antisense treatment. Mice were treated with saline, antisense, or a mismatch control (20 μg, i.c.v.) on days 1, 2, 3, and 4. On day 5, the periaqueductal gray region, an area important in μ opioid analgesia, was dissected from the animal and used in homogenate binding assays (Fig. 3). Antisense treatment (AS1) significantly decreased [3H](+)-pentazocine binding by almost 80% compared with controls (p < 0.05). There were no significant differences in binding levels between the control and mismatch groups. Thus, antisense treatment effectively down-regulated σ₁ receptor binding. The inactivity of the mismatch control supported the specificity of the approach.

Antisense treatment effectively modulated opioid analgesic activity. We also examined the antisense treatment on drugs given systemically (Fig. 4A). To examine the effects of antisense treatment, we tested treated animals with a low dose of opioid to facilitate our ability to see increased analgesic responses. Using these low doses of the μ agonist morphine, the k₁ drug U50,488H, or the k₃ agent NalBzoH, we saw significant increases in analgesic responses in antisense-treated mice. Mismatch groups were not significantly different from the control groups. Animals receiving vehicle injections showed responses virtually identical to those in the untreated control mice, ensuring that the injection paradigm itself was not responsible for these observations.

We saw similar results with supraspinal morphine and DPDPE analgesia (Fig. 4B). Again, we chose low doses of opioids that gave responses of 20% or less when given alone to control animals. Antisense treatment significantly enhanced the analgesic responses to both morphine and DPDPE. The specificity of these responses was established by the inactivity of the mismatch oligodeoxynucleotide or vehicle alone. Neither treatment significantly differed from control groups.

Previously, we demonstrated that antisense probes could be directed along the full length of the mRNA, provided care was taken when designing the probe (Standifer et al., 1994; Rossi et al., 1995). To confirm the specificity of AS1, we examined the actions of several additional antisense oligodeoxynucleotides targeting the σ₁ receptor on the analgesic activity of the k₁ drug U50,488H and the k₃ agent NalBzoH. Demonstrating that additional antisense probes targeting the same mRNA had activities similar to AS1 would confirm that the behavioral actions of AS1 were due to a down-regulation of the σ₁ receptor and not a nonspecific action of the AS1 sequence. All three antisense probes targeting the σ₁ receptor mRNA enhanced κ analgesia equally well whereas the mismatch control was inactive. Both AS2 and AS3 effectively enhanced U50,488H analgesia as effectively as AS1, and AS2 was as active as AS1 against NalBzoH analgesia (Fig. 5). The activity of multiple antisense probes argued strongly for the specificity of the response.

Although CD-1 and BALB-c mice show similar sensitivities toward morphine, the BALB-c mice are far less sensitive toward κ drugs (Chien and Pasternak, 1994). The k₁ drug U50,488H is over 3-fold less potent in BALB-c mice than in CD-1 mice (ED₅₀ values of 16.9 and 4.8 mg/kg, respectively).
In our tail-flick paradigm, NalBzoH elicits less than a 15% response in BALB-c mice at a dose approximately twice the ED$_{50}$ value in CD mice. This difference in sensitivity toward $\kappa$ drugs between the CD-1 and the BALB-c mice was lost in the presence of haloperidol, suggesting that the differences resulted from variations in the level of tonic $\sigma_1$ receptor activity (Chien and Pasternak, 1993, 1994, 1995b). To confirm the role of the $\sigma_1$ receptor in the actions of haloperidol in BALB-c mice, we next examined the effects of a $\sigma_1$ receptor antisense in BALB-c mice (Fig. 6). Intracerebroventricular administration of the $\sigma_1$ antisense AS1 enhanced systemic morphine, U50,488H, and NalBzoH analgesia (Fig. 6A). Antisense treatment markedly increased the analgesic response of low doses of supraspinal morphine or DPDPE in the BALB-C mice ($p < 0.001$) (Fig. 6B).

**Discussion**

Previously, we observed strong interactions between the $\sigma_1$ receptor system and opioid analgesia (Chien and Pasternak, 1994, 1995a,b). In these studies, systemic (+)pentazocine, a selective $\sigma_1$ receptor ligand diminished opioid analgesia, whereas systemic haloperidol potentiated it (Chien and Pasternak, 1994). The anti-opioid actions of the $\sigma_1$ receptor system were restricted to opioid analgesia, with (+)pentazocine having no appreciable effect upon morphine-induced inhibition of gastrointestinal transit (Chien and Pasternak, 1994).

The current studies further defined the actions of (+)pentazocine. Alone, (+)pentazocine had no effect when given spinally or supraspinally. When given supraspinally, (+)pentazocine had anti-opioid actions against $\mu$, $\delta$, $\kappa_1$, and $\kappa_3$ analgesia. In contrast, spinal morphine analgesia was not influenced by (+)pentazocine given either supraspinally or spinally. This observation was not anticipated since systemic (+)pentazocine reverses spinal morphine analgesia (Chien and Pasternak, 1994). The reasons underlying the difference in sensitivity of spinal morphine analgesia to systemic as opposed to either supraspinal or spinal (+)pentazocine are not clear. It may require simultaneous administration of (+)pentazocine to both sites for activity or it may also involve other sites of action, perhaps even peripheral ones.

The cloning of the $\sigma_1$ receptor (Kekuda et al., 1996; Pan et al., 1998; Seth et al., 1998) has not resolved the question of its function at the molecular level. A recent report has found a physical association of the $\sigma$ receptor with ankyrin (Hayashi and Su, 2001), but even here the functional significance of this association is unclear. However, cloning the protein opens a number of possibilities to explore its pharmacology at the molecular level using antisense approaches (Standifer et al., 1994, 1995; Pasternak and Standerfer, 1995). In the current studies, antisense treatments produced a down-regulation of the $\sigma_1$ receptor at the protein level, as shown by approximately an 80% decrease in $[^3]$H(+)-pentazocine binding compared controls, with no change in binding in mismatch-treated animals. This decreased receptor binding corresponded well to the decrease in mRNA that we reported earlier (Pan et al., 1998), confirming that the antisense treatment down-regulates both mRNA and protein. Functionally, the supraspinal antisense treatment potentiated $\mu$, $\delta$, $\kappa_1$, and $\kappa_3$ opioid analgesia. Furthermore, multiple antisense probes targeting the $\sigma_1$ receptor gave similar results against the $\kappa$.
analgesics. Thus, the actions of the antisense were not dependent upon a single sequence, further supporting the specificity of the response.

Haloperidol potentiates opioid analgesia both in patients (Maltbie et al., 1979) and rodents (Chien and Pasternak, 1993, 1994, 1995a,b). However, haloperidol is not selective. In addition to its high affinity for the σ1 receptor, haloperidol also binds dopamine D2 receptors equally well. Thus, the actions of haloperidol might be due to activity at either, or both, of these receptors. The possibility of a combined mechanism of action is more intriguing in view of studies on a D2 receptor knockout mouse (King et al., 2001). In these studies, opioid analgesia was enhanced in D2 receptor knockout mice. A similar potentiation was produced by the D2 receptor antagonist sulpiride in wild-type, but not knockout, mice. It is not clear why an earlier study failed to detect an effect of sulpiride on opioid analgesia, but it may be due in part to using different strains of mice. These D2 receptor knockout animals also confirmed the presence of an independent σ1 receptor system. (+)-Pentazocine retained its ability to reduce opioid analgesia in these knockout mice. Haloperidol still potentiated opioid analgesia in the D2 receptor knockout when given alone and reversed the actions of (+)pentazocine in the knockout mice, further establishing its role as a σ1 receptor antagonist. Together with the current studies, these observations argue strongly for independent σ1 and D2 receptor modulation of opioid analgesia.

The activity of the σ1 anti-opioid system varies among strains, possibly explaining some of their sensitivity differences to opioids (Chien and Pasternak, 1994). For example, κ drugs are far less effective analgesics in BALB-c mice than CD-1 mice. The κ1 drug U50,488H is 6-fold more potent in CD-1 mice, and the κ3 antisense NaBzoH shows a similar difference. Yet, co-administration of haloperidol with the opioids potentiated the analgesic responses in both strains and virtually eliminates the strain differences among the opioids (Chien and Pasternak, 1994). As in the CD-1 mice, antisense treatment significantly enhanced the opioid responses in the BALB-c mice, with responses similar to those in CD-1 animals. The similar activity of (+)-pentazocine: evidence for an anti-opioid σ1 system, Eur J Pharmacol 259: R7–R8.


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