Modulation of brainstem opiate analgesia in the rat by sigma_1 receptors:

A microinjection study

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Sigma₁ receptors have been implicated in the modulation of opioid analgesia. In the current study we examined the role of sigma₁ systems in the periaqueductal gray (PAG), the rostroventral medulla (RVM) and the locus coerules (LC) of the rat, regions previously shown to be sensitive to morphine. Morphine was a potent analgesic in all three regions. Co-administration of the sigma₁ agonist (+)pentazocine diminished the analgesic actions of morphine in all three regions, although the PAG was far less sensitive than the other two. Blockade of the sigma₁ receptors with haloperidol in the RVM markedly enhanced the analgesic actions of co-administered morphine, implying a tonic activity of the sigma₁ system in this region. This effect was mimicked by downregulation of RVM sigma₁ receptors using an antisense approach. However, no tonic sigma₁ activity was observed in either the LC or the PAG. The RVM also was important in modulating analgesia elicited from morphine microinjected into the PAG. The analgesic actions of morphine given into the PAG could be attenuated by (+)pentazocine placed into the RVM while haloperidol in the RVM enhanced PAG morphine analgesia. These studies illustrate the pharmacological importance of sigma₁ receptors in the brainstem modulation of opioid analgesia.
Introduction

Sigma receptors are unique proteins of approximately 28 kDaltons that have been cloned from guinea pigs (Hanner, et al., 1996), humans (Kekuda, et al., 1996), mice (Pan, et al., 1998) and rats (Mei and Pasternak, 2001; Seth, et al., 1998) with distinct pharmacological characteristics (Martin, et al., 1976; Matsumoto, 2007; Bowen, 2000a). They have been implicated in a wide range of actions. They have been associated with potassium channels (Aydar, et al., 2002) and aspects of cell proliferation and cancer (Aydar, et al., 2004; Casellas, et al., 2004; Bowen, 2000b). Among their actions, sigma receptors comprise a tonically active anti-opioid system (Mei and Pasternak, 2002; King, et al., 1997; Chien and Pasternak, 1995b; Chien and Pasternak, 1995a; Pasternak, 1994; Chien and Pasternak, 1994; Chien and Pasternak, 1993). The sigma₁ antagonist haloperidol greatly potentiated systemic opioid analgesia. Although (-)pentazocine is an effective opioid with activity at both mu and kappa receptors, (+)pentazocine, on the other hand, has no opioid activity but is a potent sigma₁ agonist. (+)Pentazocine reduced systemic opioid analgesia for mu, delta, kappa₁, kappa₃ and orphanin FQ/nociceptin ligands. This enhanced activity of opioid actions with haloperidol implied that the sigma₁ system was tonically active. Furthermore, the differences in sensitivity to opioids among several strains of mice could be eliminated by blocking sigma₁ actions with haloperidol, raising the possibility that these sensitivity differences might reflect varying levels of tonic activity of the sigma₁ system.

The cloning of sigma₁ receptors facilitated their study at the molecular level. Downregulation of sigma₁ receptors in either the mouse or rat using antisense techniques
had effects similar to those of the antagonist haloperidol. Thus, the evidence implicating the ability of sigma$_1$ receptors to modulate opioid analgesia is strong.

A number of brainstem structures have shown potent morphine analgesic activities (Bodnar, et al., 1988; Rossi, et al., 1993; Rossi, et al., 1994b; Pert and Yaksh, 1974). These studies revealed complex synergistic interactions among three morphine sensitive sites, the periaqueductal gray (PAG), the locus coeruleus (LC) and the rostroventral medulla (RVM). Autoradiographic studies indicate that sigma$_1$ receptors are present within the brainstem, including these morphine sensitive sites (Walker, et al., 1992). Furthermore, the sigma$_1$ receptor antagonist haloperidol and agonist (+)pentazocine, influence supraspinal, but not spinal, morphine analgesia. (JF Mei and GW Pasternak, unpublished data). This supraspinal modulation of opioid analgesia by sigma$_1$ receptors raises questions regarding the regional localization of these interactions. We now report the mapping of rat sigma$_1$ receptor modulation of morphine analgesia in brainstem nuclei.
Materials and Methods

Materials

Na$^{125}$I was purchased from New England Nuclear Corporation (NEN; Boston, MA, U.S.A.). $^{125}$I(+)Pentazocine labeling was performed using the chloramine T/sodium metabisulfate method (Letchworth, et al., 2000). (+)Pentazocine and morphine sulfate were gifts from the Research Technology Branch of NIDA. Halothane was purchased from Halocarcon Laboratories (Hackensack, NJ, U.S.A.). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). All the drugs used in the in vivo studies were dissolved in saline. Glass fiber filters (#32) were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). Formula 963 liquid scintillant was purchased from New England Nuclear Corporation (Boston, MA, U.S.A.).

Animals and analgesic testing

Male albino Sprague-Dawley rats (175 – 250 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and maintained on a 12 hour light/dark cycle with rodent chow and water available ad libitum. Rats were housed in groups of two in clear plastic cages at ambient room temperatures between 21 °C and 25 °C until they were tested. All animal studies were approved by the IACUC of the Memorial Sloan-Kettering Cancer Center and adhere to NIH guidelines.

Analgesia was determined using the radiant heat tail-flick technique as previously reported (Chien and Pasternak, 1995b). The thermal stimulus was positioned 8 cm above the dorsum and 3–9 cm proximal to the tip of the tail of a lightly restrained animal. The mean of two latency readings were taken for each animal at each indicated time. The
baseline latencies ranged between 2-3 sec and maximal latency of 10 seconds was used to minimize the tissue damage. Studies employed groups of 4-7 rats. Saline injections did not appreciably alter the latencies over time compared with the baseline value, as shown in Fig. 2a. The Percent Maximal Percent Effect (%MPE) was calculated as the (observed latency-baseline latency)/(maximal latency-baseline latency).

Cannulations

Rats were anesthetized with chlorpromazine HCl (CPZ) (3mg/kg, i.p.) given 20 minutes before ketamine HCl (100 mg/kg, i.m.) injection. The animal was set on the Kopf Stereotaxic Instrument and a stainless-steel guide cannula (26 ga, Plastic One Products) was implanted stereotaxically in the specified location. Cannulae coordinates were the same as prior studies (Rossi, et al., 1993). Cannulae were secured to the skull with three anchor screws and dental acrylic. The cannulae were then capped with a dummy cannulae (Plastic One Products). Each animal was housed in a single cage and allowed one week to recover from surgery before behavioral testing began. All cannulated rats were tested with morphine to confirm cannualae placement at least 5 days before the experiment: PAG, 2.5 µg; RVM and LC 5 µg. Animals displaying a poor response were assumed to have a faulty cannualae placement and were not used in further experiments (Bodnar, et al., 1988; Bodnar, et al., 1991).

After behavioral testing, cannulae placement was confirmed anatomically. Rat brains were fixed in 10% buffered formalin overnight and transferred into 30% sucrose until section cutting. The brains were cut coronally, stained with Cresyl violet and placement confirmed by light microscopy. Animals whose placements were inaccurate
were not included in the result (Bodnar, et al., 1988; Bodnar, et al., 1991; Rossi, et al., 1993).

**Antisense and in vivo assay**

Antisense oligodeoxynucleotides were designed using the rat sigma1 receptor cDNA sequences with the ALIGN and GENE RUNNER programs (Scientific and Educational Software) and were purchased from Midland Certified Reagent Co. (Midland, TX, U.S.A.). The oligodeoxynucleotides were repurified using sodium acetate precipitation and dissolved in saline to make a final concentration of 5 µg/µl.

The antisense oligodeoxynucleotide sequence (RS357AN) corresponding to the cloned rat sigma1 receptor (rs2-2) was 5’-CCAGCCGCRCGCACGTTAC-3’, and the mismatch control differed by switching 6 bases, 5’-CCACGCGGCCGCGTTACC-3’.

Groups of mice were treated with antisense oligoes (10 µg in 2 µl saline per injection) or vehicle (saline) on days 1, 2, 4, and tested for analgesia on day 5 with morphine sulfate (mu). Previous studies in rats and mice have shown that antisense treatment can downregulate opioid or sigma1 receptors at both the mRNA and protein levels (Rossi, et al., 1994a; Mei and Pasternak, 2001).

**Data Analysis**

Rat analgesia data were compared by both the area under the time-action curve and at peak effect using one way ANOVA followed by a post-hoc Tukey analysis. EDs0 values were calculated by Pharm/PCS software using nonlinear regression analysis.
Results are presented as means ± S.E.M. of triplicate experiments unless specified otherwise.
Results

Modulation of supraspinal morphine analgesia by a sigma₁ receptor agonist

Microinjection of morphine into any one of a number of sites within the rat brain elicits a profound naloxone-sensitive analgesia (Pert and Yaksh, 1974; Bodnar, et al., 1988). We chose three sites sensitive to morphine analgesia to explore the interaction between supraspinal opioid and sigma₁ systems: the periaqueductal gray (PAG), the locus coeruleus (LC) and the rostroventral medulla (RVM).

Microinjection of morphine into each of the three regions produced a dose-dependent analgesia, with peak effects of 30 min in the PAG and the RVM and 15 min in the LC and durations of action between 60 and 90 min (Fig. 1). These results are consistent with our earlier studies (Rossi, et al., 1993).

We next explored whether sigma₁ systems modulated morphine action in these regions. The sigma₁ agonist (+)pentazocine significantly blocked morphine analgesia in the PAG, RVM and LC (Fig. 2a-c) in a dose-dependent manner (Fig. 2d), but the sensitivity of the regions varied markedly. The RVM was the most sensitive to (+)pentazocine, with an ID₅₀ of 2.6 ng. The LC also was quite sensitive, with an ID₅₀ of 17.4 ng. In contrast, the doses of (+)pentazocine needed to reduce morphine analgesia in the PAG were up to 1000-fold greater, with an ID₅₀ of 4090 ng (Fig. 2d).

In both the PAG site and RVM sites, the effects of (+)pentazocine could be overcome by increasing the morphine dose (Fig. 3; Table 1). In the PAG, (+)pentazocine significantly shifted the morphine dose-response curve over 4-fold, increasing the ED₅₀ from 1.5 to 6.5 µg. We observed a similar significant shift in the morphine dose-response curve in the RVM, with the morphine ED₅₀ significantly increasing from 2.8 to
12 µg. In both regions, morphine retained the ability to fully overcome the inhibitory actions of (+)pentazocine.

Modulation of PAG analgesia through RVM sigma₁ receptor systems

Opioid administration into the PAG activates a circuit between the PAG and the RVM, with release of endogenous opioids (Osborne, et al., 1996; Fields, 2000). We therefore examined potential interactions between the PAG and RVM. Administration of (+)pentazocine into the RVM attenuated the analgesic activity of morphine microinjected into the PAG (Fig. 4a), consistent with the prior demonstration of the circuit. (+)Pentazocine was equally effective in attenuating the actions of morphine given into either the PAG or RVM (Fig 4b). The ability of RVM (+)pentazocine to block PAG morphine analgesia is further supported by the sensitivity of PAG morphine analgesia to RVM (+)pentazocine at only 50 ng, a dose that was inactive against morphine when both were given directly into the PAG.

This ability of (+)pentazocine administered into the RVM to block PAG morphine analgesia raised the question of whether the high doses of (+)pentazocine needed to lower morphine analgesia when both drugs were given into the PAG might be due to diffusion of (+)pentazocine from the PAG to the RVM. However, this appears to be unlikely for several reasons. Autoradiographically, we failed to see evidence of [¹²⁵I](-)-pentazocine diffusing in the RVM following its injection into either the LC or PAG after 45 minutes. However, the sensitivity of autoradiography is limited and might not be sufficient to detect (+)pentazocine doses necessary for activity in the PAG and RVM. We therefore examined the ability of the sigma₁ antagonist haloperidol to reverse (+)pentazocine
actions. If the activity of PAG (+)pentazocine was due to its diffusion into the RVM, haloperidol administered into the RVM should reverse (+)pentazocine’s actions and increase morphine analgesia. Haloperidol did not reverse (+)pentazocine (Fig. 5), making it unlikely that the actions of (+)pentazocine in the PAG result from diffusion into the RVM. However, it leaves open the question of why this region is so insensitive, particularly since it has readily demonstrable levels of sigma binding sites autoradiographically (Gundlach, et al., 1986).

Modulation of supraspinal morphine analgesia by a sigma$_1$ receptor antagonist

Haloperidol enhances opioid analgesia, presumably by blocking a tonically active sigma$_1$ system (Chien and Pasternak, 1993; Chien and Pasternak, 1994; Chien and Pasternak, 1995b; King, et al., 1997; Mei and Pasternak, 2002). To assess the possibility of tonic sigma$_1$ activity within individual brainstem regions, we examined the effects of the sigma$_1$ antagonist haloperidol on morphine analgesia in each region. At the maximal doses we could test due to solubility constraints (5 µg), haloperidol had little effect on morphine analgesia when both were microinjected into either the PAG or the LC (Fig. 6). However, haloperidol co-administered with morphine into the RVM markedly enhanced the analgesic response. Full dose-response curves revealed that haloperidol shifted morphine’s ED$_{50}$ in the RVM by approximately 2-fold (Fig. 7; Table 1). These findings confirm tonic sigma$_1$ activity in the RVM, but not in the PAG and LC sites. Haloperidol can interact with both sigma$_1$ and dopamine D2 receptors. To ensure that the actions of haloperidol were mediated through sigma$_1$ sites, we also tested (-)sulpiride, a D2 receptor-selective antagonist. Administering (-)sulpiride along with morphine into the
RVM failed to enhance the analgesic actions of morphine in the RVM, supporting the suggestion that haloperidol was acting through blockade of sigma\textsubscript{1} receptors (JF Mei and GW Pasternak, unpublished observations), results similar to those in mice (Chien and Pasternak, 1994).

**Effects of downregulation of sigma\textsubscript{1} receptors on morphine analgesia in the RVM**

Antisense approaches have proven valuable in selectively downregulating opioid receptors and sigma receptors within the central nervous system, with a selectivity greater than most antagonists (Standifer, et al., 1994; Pasternak and Pan, 2000). To confirm the role of sigma\textsubscript{1} receptors in the potentiation of morphine analgesia, we therefore utilized an antisense approach similar to one used to downregulate mu opioid receptors (Rossi, et al., 1994a). In earlier work our group demonstrated the ability of antisense treatment to downregulate sigma\textsubscript{1} receptors and potentiate opioid analgesia in both mice and rats (Pan, et al., 1998; Mei and Pasternak, 2002; Mei and Pasternak, 2001). In this study, we administered the antisense directly into RVM and tested morphine given into the same region. Antisense treatment enhanced morphine analgesia in the RVM (Fig. 8), as shown both by the peak effect in the time-action curve (p<0.001) and by the area under the curves ((p<0.001). To ensure the specificity of the effect, we used a mismatch antisense probe in which the order of three sets of adjacent bases were switched, keeping the total base composition the same. The mismatch was without effect, confirming the selectivity of the antisense effect.

Sigma\textsubscript{1} actions in the RVM can modulate the analgesic actions of morphine in the PAG. We therefore examined of the effects of antisense treatment in the RVM on the
analgesic activity of morphine administered into the PAG (Fig. 8). Morphine in the PAG at a dose that failed to significantly elevate tailflick latencies above baseline levels elicited a profound analgesic response following antisense treatment in the RVM. Again, the inactivity of the mismatch antisense oligodeoxynucleotide confirmed the selectivity of the response. These antisense studies confirmed the role of sigma1 receptors in the modulation of morphine analgesia and the ability of sigma1 receptors within the RVM to modulate the analgesic actions of PAG morphine.
Discussion

Within the brain stem region, microinjection studies have identified three morphine sensitive regions, including the periaqueductal gray (PAG), locus coeruleus (LC) and rostroventral medulla (RVM) (Jacquet and Lajtha, 1973; Pert and Yaksh, 1974; Bodnar, et al., 1988; Bodnar, et al., 1991; Rossi, et al., 1993). Although each can induce analgesia alone, they also interact with each other, as shown by analgesic synergy between morphine administered in the PAG and the RVM. (Rossi, et al., 1994b). This pharmacological interaction is consistent with detailed studies demonstrating the importance of the RVM in mediating the actions of opioids in the PAG (Fields, 2000; Christie, et al., 2000; Osborne, et al., 1996).

The sigma receptor system reportedly plays an important role in anti-opioid, antipsychotic, neuroprotective, and anti-depressant activities (Bowen, 2000a; Hayashi and Su, 2005). (+)Pentazocine, a selective sigma1 receptor agonist, blocks opioid analgesia while the sigma1 antagonist haloperidol potentiates opioid analgesia (Chien and Pasternak, 1993; Chien and Pasternak, 1994; Chien and Pasternak, 1995b; Chien and Pasternak, 1995a), results supported by antisense approaches in mice (Mei and Pasternak, 2002; Mei and Pasternak, 2001). The object of the current study was to explore the role of sigma1 receptor systems within a series of brainstem nuclei of the rat known to be important in opioid analgesia.

The ability of the sigma1 agonist (+)pentazocine to diminish opioid analgesia in all three brainstem regions clearly demonstrates the presence and potential importance of the sigma1 system in the modulation of opioid mechanisms. However, there were significant differences among the sites. (+)Pentazocine was quite potent in both the LC
and the RVM, but not the PAG despite the high levels of sigma1 binding as assessed autoradiographically (Gundlach, et al., 1986). Indeed, the doses necessary to influence morphine analgesia in the PAG were several orders of magnitude greater than the other two regions. Initially, we assumed that this might reflect the need for the drug to diffuse from the PAG to the RVM, particularly since (+)pentazocine at far lower doses administered directly into the RVM attenuated morphine analgesia following morphine injection into the PAG. However, several lines of evidence suggest that this is not likely. First, directly looking at the diffusion of [125I]pentazocine failed to show any appreciable levels in the RVM after administering the compound into the PAG. In addition, the sigma1 antagonist haloperidol given into the RVM did not reverse the actions of (+)pentazocine administered into the PAG, which would have been expected if the (+)pentazocine were acting in the RVM. Yet, it still not clear why (+)pentazocine is so weak in the PAG. It may simply reflect the limitations of the sigma1 system in this region in modulating analgesia, but at these high doses (+)pentazocine may be acting through alternative, non-sigma mechanisms.

Although morphine analgesia in both the LC and RVM was lowered by low doses of (+)pentazocine, implying a highly sensitive sigma1 system, only the RVM appeared to have tonic sigma1 activity based upon the ability of the sigma1 antagonist haloperidol to enhance morphine actions. Earlier work in mice (Chien and Pasternak, 1995a; Chien and Pasternak, 1994; Chien and Pasternak, 1993; King, et al., 1997; Mei and Pasternak, 2002) and in rats (Chien and Pasternak, 1995b; Mei and Pasternak, 2001) showed that haloperidol enhanced opioid analgesia. Interestingly, varying levels of tonic sigma1
receptor activity appeared to be responsible for many of the differences in sensitivities of some strains to opioids.

Although haloperidol is a potent sigma₁ antagonist, it also blocks dopamine D2 receptors quite effectively, making the interpretation of some of the studies difficult. Unlike haloperidol, the selective dopamine D2 receptor antagonist (-)sulpiride does not interact with sigma₁ receptors. In our prior studies in CD-1 mice, (-)sulpiride did not reproduce the actions of haloperidol. However, in other mouse strains and models, dopamine D2 receptors can influence opioid action (Calcutt, et al., 1971; Kamei and Saitoh, 1996; Kunihara, et al., 1993), a finding that was supported in a more recent study looking at opioid actions in a dopamine D2 receptor knockout mouse (King, et al., 2001). Although there may be situations in which D2 receptors modulate opioid actions, sigma₁ systems also are important. This was clearly shown by the ability of haloperidol to potentiate and (+)pentazocine to block opioid analgesia in the D2 knockout mice. Since these mice have no D2 receptors, the actions must be mediated through sigma₁ sites.

To further verify the role of sigma₁ receptors in the RVM we also examined the effects of antisense. Antisense approaches can effectively downregulate receptors and influence opioid behavior (Standifer, et al., 1994). Downregulation of sigma₁ receptors in the RVM by antisense, but not by mismatch controls, potentiated morphine analgesia in much the same way as haloperidol. This confirms the role of sigma₁ receptors in the RVM in morphine analgesia.

The interactions between the PAG and RVM were particularly interesting. A large literature has established the importance of the PAG to RVM pathway in opioid analgesia (Osborne, et al., 1996; Fields, 2000). This is further supported by the
synergistic opioid interactions in microinjection studies (Rossi, et al., 1993). The current studies support these PAG/RVM interactions. (+)Pentazocine administered into the RVM attenuated morphine analgesia from the PAG while haloperidol in the RVM enhanced it. Finally, antisense treatment targeting sigma₁ receptors in the RVM potentiated morphine analgesia from the PAG, much like the actions of haloperidol. Thus, the sigma₁ system in the RVM also modulates opioid actions from the PAG.

In conclusion the current studies support a role for sigma₁ receptor actions in the brainstem. Its activity within the three regions differed widely. Whereas both the LC and RVM were sensitive to (+)pentazocine, the PAG was not, despite the demonstration of sigma₁ receptors in this site autoradiographically (Walker, et al., 1992). The RVM was particularly interesting in that it was the only regions with evidence for tonic sigma₁ activity and it could modulate the analgesia from morphine given into the PAG.
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Rossi GC, Pasternak GW and Bodnar RJ (1994b) μ and δ opioid synergy between the periaqueductal gray and the rostro-ventral medulla. *Brain Res* **665**:85-93.


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Legends for Figures

Fig. 1. Morphine analgesia in the periaqueductal gray, locus coeruleus and rostroventral medulla

Cannulated rats received the stated morphine dose and were tested at the indicated time using the tail-flick assay. Baseline tail-flick latencies were taken prior to the experiments. Data were compared by the areas under the time-action curve. A, PAG (n=14); B, LC (n=7); C, RVM (n=7). The peak effects of morphine PAG and RVM analgesia were at 30 min while that of morphine LC analgesia was at 15 min. In the PAG, LC and RVM the peak effects of the different morphine doses were significantly different from each other (P<0.001).

Fig. 2. Effect of (+)pentazocine on morphine analgesia in brainstem sites

A: Groups of rats cannulated in the PAG received either saline (n=7), morphine alone (2.5 µg; n=7) or morphine (2.5 µg) in combination with (+)pentazocine (625 ng, 1250 ng, 5000 ng or 6500 ng) and analgesia assessed at the indicated time. Significant differences in the areas under the time-action curves were observed (P < 0.001) and at peak effect (:P < 0.001). B: Groups of rats cannulated in LC received either morphine alone (5 µg, n=4) or morphine (5 µg) in combination with (+)pentazocine (2.5 ng, 10 ng, 50 ng or 100 ng and analgesia assessed at the indicated time. Significant differences in the areas under the time-action curves were observed (P < 0.001) and at peak effect (P < 0.001). C: Groups of rats cannulated in the RVM received either morphine alone (10 µg, n=7) or morphine (10 µg) in combination with (+)pentazocine (2.5 ng, 10 ng or 25 ng)
and analgesia assessed at the indicated time. Significant differences in the areas under the time-action curves were observed ($P < 0.02$) and at peak effect ($P < 0.001$).  

**D:** The dose-response relationship of (+)pentazocine on morphine analgesia was assessed at peak effect in each of the regions noted. Analgesia is shown as %MPE to facilitate comparisons among regions. ID$_{50}$ values with 95% confidence limits were 4090 ng (830, 5470) in the PAG, 17.4 ng (9.1, 25) in the LC and 2.6 ng (0.1, 5.3) in the RVM.

**Fig. 3. The effect of (+)pentazocine on morphine analgesic dose-response curves in the PAG and RVM**

**A:** Groups of rat cannulated in the PAG (n=7) received the indicated morphine dose in the absence or presence of (+)pentazocine (6.5 µg) and analgesia assessed at peak effect. (+)Pentazocine significantly shifted the dose-response curve ($P<0.05$). The ED$_{50}$ value and 95% confidence limits was 1.5 µg (1.3, 1.7) for morphine alone and 6.5 µg (4.4, 9.6) for morphine in combination with (+)pentazocine.

**B:** Groups of rats cannulated in the RVM (n=7) received the indicated morphine dose in the absence or presence of (+)pentazocine (25 ng) and analgesia assessed at peak effect. (+)Pentazocine significantly shifted the dose-response curve ($P<0.05$). The ED$_{50}$ value and 95% confidence limits was 2.8 µg (2.3, 3.6) for morphine alone and 12 µg (7, 23) for morphine in combination with (+)pentazocine.

**Fig. 4. Interactions between the RVM and PAG.**

**A)** Groups of rats (n=4) cannulated in both the RVM and the PAG received morphine (2.5 µg) in the PAG 10 min after either saline or (+)pentazocine (50 ng) in the
RVM and analgesia was assessed at the indicated time after the morphine. Significant differences in the areas under the time-action curves were observed ($P < 0.05$) as well as comparison of peak effect ($P < 0.001$). B) Groups of rats cannulated in both the RVM and the PAG received morphine (2.5 µg) in the PAG and the indicated doses of (+)pentazocine in the RVM. Results are peak effects from each time-action curve. Results are presented as %MPE to facilitate comparisons.

**Fig. 5. Site of action of PAG (+)pentazocine**

Groups of rats cannulated in the PAG (n=7) received morphine (2.5 µg) alone or in combination with (+)pentazocine 6.5 µg and analgesia assessed at the indicated time. Another group of rats cannulated in both the PAG and RVM (n=5) received both morphine and (+)pentazocine in the PAG and either saline or haloperidol (5 µg) in the RVM and analgesia assessed at the indicated time. Compared to the morphine alone group, (+)pentazocine significantly blocked morphine PAG analgesia with or without pretreatment of haloperidol at RVM site, assessed either at peak effect (30min, $P < 0.001$) or by the areas under the time-action curves ($P < 0.001$). The two groups with or without haloperidol pretreatment at RVM site showed no significant differences in the areas under the time-action curves.

**Fig. 6: Effects of haloperidol on morphine analgesia in brainstem regions**

A) Rats cannulated in the RVM received either vehicle (n=6) or haloperidol (5 µg; n=6) 10 minutes prior to morphine (1.5 µg). Haloperidol significantly enhanced the response ($P < 0.001$). B and C) Groups of rats with cannulae in either the PAG, RVM
or LC received morphine 10 min after either saline or haloperidol (5 µg) and a time-action curve for analgesia was obtained. Peak effects were determined for each group (B) and areas under the curve calculated (C). Neither the PAG site nor the LC displayed significant differences with haloperidol treatment with either measure. However, haloperidol in the RVM significantly enhanced morphine analgesia assessed as either peak effect ($P < 0.001$) or area under curve ($P < 0.05$).

**Fig. 7: Effect of haloperidol on morphine dose-response curves in the RMV**

Groups of rats cannulated in the RVM (n=4) received either vehicle or haloperidol (5 µg) 10 minutes prior to injection of the stated dose of morphine. Results are from peak effects, determined at 30 min after the morphine injection. Morphine alone had an ED$_{50}$ value of 3.8 (3.2, 4.5) µg. Inclusion of haloperidol lowered the ED$_{50}$ value to 1.5 (1.4, 1.7) µg.

**Fig. 8: Effects of antisense targeting the sigma$_1$ receptor on morphine analgesia in the RVM on either RVM or PAG morphine**

Groups of rats cannulated in the RVM received either saline (n=6), rat sigma$_1$ antisense (RS357AN, 10µg, n=8) or the mismatch oligodeoxynucleotide (RS357MIS, 10µg, n=5) on days 1, 2 and 4. On day 5, all animals received morphine RVM (1 µg) and analgesia was assessed. Another set of groups of rats were cannulated in both the RVM and PAG and administrated either saline (n=5), rat sigma$_1$ antisense (RS357AN, 10µg, n=6) or the mismatch oligodeoxynucleotide (RS357MIS, 10µg, n=4) in the RVM on days 1, 2 and 4 and on day 5 all animals received morphine in the PAG (1 µg) and analgesia
was assessed over time. Antisense treatment in the RVM significantly decreased morphine analgesia following administration in either the RVM or the PAG when assessed either as peak effect or as the area under the curve ($P < 0.001$).
Table 1: Sigma₁ receptor and morphine PAG and RVM analgesia.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>PAG</th>
<th>RVM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>1.5 (1.3, 1.7)</td>
<td>3.8 (3.2, 4.5)</td>
</tr>
<tr>
<td>Morphine + Haloperidol</td>
<td>Not Determined</td>
<td>1.5 (1.4, 1.7)</td>
</tr>
<tr>
<td>Morphine + (+)Pentazocine</td>
<td>6.5 (4.4, 9.6)</td>
<td>12 (7, 23)</td>
</tr>
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Group of rats (n ≥ 4) were pretreated with either vehicle, haloperidol (5 µg) or (+)pentazocine (6.5 µg) 10 minutes prior to injection of at least three different doses of morphine in either the PAG or the RVM site, and analgesia was assessed in the tailflick assay. ED₅₀ with 95% confidence limits were calculated and presented. The ED₅₀ value of morphine PAG analgesia with haloperidol pretreatment was not determined because no significant change compared to naïve animals was seen in single dose studies.
Figure 2
Figure 3

A. PAG

- • Morphine alone
- ○ Morphine with (+)Pentazocine

Latency (sec)

Morphine Dose (μg)

B. RVM

- • Morphine alone
- ○ Morphine with (+)Pentazocine

Latency (sec)

Morphine Dose (μg)
Figure 4

A

- Morphine Alone (PAG)
- Morphine (PAG) and (+)Pentazocine (RVM)

Latency (sec)

Time (hr)

B

- RVM Pentazocine with
- RVM Morphine
- PAG Morphine

Analgesia (%MPE)

(+)-Pentazocine Dose (ng)
Figure 5

- PAG: Morphine Alone
- PAG: Morphine with (+)Pentazocine
- PAG: Morphine with (+)Pentazocine
- RVM: Haloperidol

Latency (sec) vs Time (hr)
Figure 7

- ○ Morphine alone
- ● Morphine with Haloperidol

Latency (sec)

RVM

Morphine Dose (μg, RVM)
Figure 8

A

RVM Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>Mismatch</th>
<th>Antisense</th>
</tr>
</thead>
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<td>RVM morphine</td>
<td><img src="image1" alt="Saline" /></td>
<td><img src="image2" alt="Mismatch" /></td>
<td><img src="image3" alt="Antisense" /></td>
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Peak Tail-flick Latencies (sec)

* p < 0.001

B

RVM Treatment

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Area Under Curve

* p < 0.001