Dose-Dependent Pain-Facilitatory and -Inhibitory Actions of Neurotensin Are Revealed by SR 48692, a Nonpeptide Neurotensin Antagonist: Influence on the Antinociceptive Effect of Morphine¹, ²

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ABSTRACT
Neurotensin has bipolar (facilitatory and inhibitory) effects on pain modulation that may physiologically exist in homeostasis. Facilitation predominates at low (picomolar) doses of neurotensin injected into the rostroventral medial medulla (RVM), whereas higher doses (nanomolar) produce antinociception. SR 48692, a neurotensin receptor antagonist, discriminates between receptors mediating these responses. Consistent with its promotion of pain facilitation, the minimal antinociceptive responses to a 30-pmol dose of neurotensin microinjected into the RVM were markedly enhanced by prior injection of SR 48692 into the site (detected using the tail-flick test in awake rats). SR 48692 had a triphasic effect on the antinociception from a 10-nmol dose of neurotensin. Antinociception was attenuated by femtomolar doses, attenuation was reversed by low picomolar doses (corresponded to those blocking the pain-facilitatory effect of neurotensin) and the response was again blocked, but incompletely, by higher doses. The existence of multiple neurotensin receptor subtypes may explain these data. Physiologically, pain facilitation appears to be a prominent role for neurotensin because the microinjection of SR 48692 alone causes some antinociception. Furthermore, pain-facilitatory (i.e., antianalgesic) neurotensin mechanisms dominate in the pharmacology of opioids; the response to morphine administered either into the PAG or systemically was potentiated only by the RVM or systemic injection of SR 48692. On the other hand, reversal of the enhancement of antinociception occurred under certain circumstances with SR 48692, particularly after its systemic administration.

Recent studies have demonstrated that the tridecapeptide neurotensin that is contained in pain-modulatory neuronal projections from the PAG to the RVM (Beitz, 1982) functions not only to inhibit pain transmission (Behbehani, 1992; Clineschmidt et al., 1979; Fang et al., 1987; Kalivas et al., 1982) but also to facilitate pain transmission in a dose-dependent manner (Urban and Smith, 1993, 1994; Urban et al., 1996a). For example, high doses (nanomolar range) of the peptide microinjected into the RVM have an antinociceptive action, as shown by an increased tail-flick latency in response to a heat stimulus. In contrast, lower doses (picomolar range) in the RVM have been shown to reduce latencies in the tail-flick and hot-plate tests (Urban and Smith, 1993, 1994), facilitate spinal nociceptive unit responses to noxious heat (Urban and Gebhart, 1994) and increase the visceromotor response to noxious visceral stimulation (Urban et al., 1996b). The striking dose-dependency of neurotensin on pain modulation within the neuronal circuitry of the RVM strongly suggests that the basis for its opposing actions is separate and distinct neurotensin receptor subtypes with varying affinities for the peptide. Moreover, both of these actions of neurotensin are mediated in part by separate and distinct neuronal pathways that function to modulate pain at the spinal level. That is, the pain-facilitatory response to neurotensin is blocked by the intraspinal application of cholecystokinin receptor antagonists (Urban et al., 1996a), whereas the antinociceptive action appears to be inhibited by the depletion of spinal norepinephrine (Behbehani, 1992). It is suggested by these studies that neurotensin neurons from the PAG to the RVM function to maintain a homeostatic balance of pain modulation.
balance in animal responsivity to pain, with low doses of exogenous neurotensin favoring the pain-facilitatory function, and the antinociceptive function predominating with higher doses.

Before the demonstration of bipolar actions of neurotensin on pain modulation, it was generally assumed that neurotensin functioned solely as a mediator of pain inhibition (Clinical Schmidt et al., 1979). In fact, it was expected that neurotensin neurons from the PAG to the RVM supported the spinally directed antinociceptive response to opioids (Behbehani, 1992; Fang et al., 1987; Fields et al., 1991). However, in contrast to this expectation, Urban and Smith (1993, 1994) demonstrated a prominent antiodip role for neurotensin within the RVM, consistent with their observation of a pain-facilitating role for low doses of the peptide. They observed that when an antagonistic dose of either [D-Trp]neurotensin, a partial agonist of neurotensin receptors, or neurotensin antiserum was microinjected into the RVM of rats, the antinociceptive response to morphine sequentially administered into the PAG was greatly enhanced rather than inhibited. Moreover, Smith et al. (1995) subsequently demonstrated that antagonism of neurotensin receptors results in the potentiation of the antinociceptive response to systemically administered morphine as well. Thus, it appears that neurotensin has a prominent role in pain-modulatory circuitry as an antinociceptive neurotransmitter.

These studies of the function of neurotensin in the RVM were limited by an inability to pharmacologically resolve actions of neurotensin that may be mediated by putative subtypes of its receptors. [D-Trp]neurotensin was not useful for discriminating neurotensin receptor subtypes because of its intrinsic activity at neurotensin receptors, which limited the doses of the antagonist that could be used. In addition, because neurotensin antiserum presumably inactivates all of the biologically active neurotensin in synapses, only the predominat physiological function of neurotensin is likely to be resolved. An important recent development, therefore, was the synthesis of a potent and selective nonpeptide antagonist of the neurotensin receptor, SR 48692 [2-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)]pyrazol-3-yl]carbonylaminol]tricyclo[3.3.1.1^{3\,5}]decane-2-carboxylic acid], which lacks intrinsic activity and appears to pharmacologically discriminate between several biochemical and physiological actions mediated by neurotensin (Gully et al., 1993). For example, SR 48692 competitively inhibits intracellular Ca^{2+} influx in human colon cancer tissue, neurotensin-potentiated K^+-evoked dopamine release from guinea pig striatal slices (Gully et al., 1993), neurotensin-mediated signal transduction responses (i.e., inositol monophosphate, cGMP and cAMP formation) in mouse neuroblastoma cells and inositol monophosphate formation in human colon cancer cells (Oury-Donat et al., 1985). SR 48692 also inhibits several physiological responses to neurotensin, including dopamine-independent turning behavior induced by intrastriatal injection of neurotensin in mice (Azzi et al., 1994; Gully et al., 1993). In addition, Dubuc et al. (1994) reported that the antagonist inhibits the hypokinetik effect elicited by central administration of neurotensin in rats. In contrast, however, others describe its inability to alter the locomotor effects of a systemically active neurotensin peptide [N-MeArg-Lys-Pro-Trp-tert-Leu-Leu] (Pugsley et al., 1994). On the other hand, SR 48692 clearly discriminates between neurotensin receptors by antagonizing the behavioral changes induced by the injection of neurotensin into the ventral tegmental area or nucleus accumbens but has no effect on those neurotensin receptors, which induce changes in dopaminergic transmission in these brain regions (Steinberg et al., 1994). Moreover, Dubuc et al. (1994) reported that SR 48692 does not antagonize receptors associated with the hypothermic response of neurotensin or seem to be effective as an antagonist of neurotensin-induced antinociceptive responses. However, SR 48692 was used over a narrow dose range in the studies of neurotensin-induced antinociception, and several investigators have shown that the antagonist may produce multiphasic (i.e., bell- or U-shaped dose-response curves) dose-dependent alterations of the behavioral effects of neurotensin (Poncelet et al., 1994; Steinberg et al., 1994). Thus, a more complete evaluation of the effect of SR 48692 on nociceptive behavior using a wider dose range of the drug is required for this conclusion to be accepted.

The current study was conducted to determine whether SR 48692 would discriminate neurotensin receptors mediating antianalgesic and antinociceptive responses within the RVM of the RVM. SR 48692 was administered over a wide dose range into the RVM, and neurotensin was injected into the same brain area in one of two doses associated in previous studies with either pain facilitation or antinociception (see above). Subsequently, this same dose range of SR 48692 was injected into the RVM to determine its influence on the antinociceptive response to morphine administered either into the PAG (to alter the activity of neurotensin neuronal processes extending to the RVM) or systemically (to evaluate the influence of the PAG to RVM neurotensin neuronal pathway on the net antinociceptive response of morphine). Last, because SR 48692 is a nonpeptide antagonist with access to the CNS after systemic administration, it was administered systemically with morphine to determine if its ability to alter morphine’s antinociceptive action was retained.

**Methods**

**Animal care.** All animals were used in accordance with guidelines outlined in the United States Public Health Service publication “Policy on the Humane Care and Use of Laboratory Animals.” Experimental protocols were approved by the West Virginia University Animal Care and Use Committee. Male Sprague-Dawley rats (295–325 g, Hilltop Laboratory Animals, Scottsdale, PA) were housed in the animal facility at the Robert C. Byrd Health Sciences Center and given food and water *ad libitum*.

**Supraspinal microinjection preparation and procedure.** Procedures were conducted as previously reported (Urban and Smith, 1993). Rats were prepared with indwelling guide cannulae (a 23-gauge needle shaft) ≥7 days before use. The cannulae were stereotaxically (David Kopf stereotaxic apparatus) implanted in the skull of rats anesthetized with ketamine (120 mg/kg i.p.) and supplemented with atropine (0.4 mg/kg i.p.) to reduce secretions. Lidocaine (0.15 ml of a 0.5% solution s.c.) was infiltrated under the skin of the skull. Each cannula was fitted with a 30-gauge stainless steel stylet and kept in place with acrylic dental cement secured by skull screws.

With the rats loosely restrained in wire cages, microinjections were performed by lowering a 30-gauge needle through the guide cannula and delivering 0.5 μl of a drug solution over a 30-sec time interval. The injection rate was controlled using a Harvard model 975 infusion pump equipped with a Hamilton 10-μl syringe. To minimize the volume of drug solution needed, the syringe and a portion of the polyethylene tubing connected to the infusion needle were filled with water. A small air bubble separated the water and

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the drug solution. As defined by Paxinos and Watson (1986), the final coordinates (in mm) for the PAG were rostral caudal, +1.7 (from the intra-aural line); medial lateral, 0.7; and dorsal ventral, −6.4. For the RVM, the target site of the RMg was defined as −2.0 rostral caudal, 0 medial lateral and −8.5 dorsal ventral. Correct placement of the microinjection cannulae was verified in each animal by removing and treating the brain overnight in 10% formalin and then examining cryostat sections.

Experiments to evaluate the interaction between SR 48692 (dissolved in DMSO) and neurotensin (dissolved in saline) were performed by microinjecting the antagonist followed by an injection of neurotensin into the same site. The interval between the injection of SR 48692 and neurotensin was 20 min to minimize the influence of fluctuations in tail temperature on the pain-modulatory response to the peptide (see Results, table 1). A sequential application of SR 48692 into the RVM and morphine (dissolved in saline) into the PAG was also performed to evaluate the influence of neurotensin neuronal projections from the PAG-RVM on the antinociceptive effect of morphine. The interval between the injection of SR 48692 and morphine was 10 min. In experiments in which the influence of systemic administration of the SR 48692 on the antinociceptive effect of morphine was evaluated, the antagonist was mixed in two drops of Tween 80 and subsequently suspended in distilled water for intraperitoneal injection. The interval between the injection of SR 48692 and either PAG or systemic administration of morphine was 30 min, which corresponds to the time used in studies performed by Ponecet et al. (1994).

**Antinociceptive testing.** The threshold to thermal nociceptive stimuli were measured using modified versions of the tail-flick test (D’Amour and Smith, 1941) and a model 33 Analgesia Meter (ITC, Woodland Hills, CA). The time for the rat to remove its tail from the path of the focused light source was expressed as the TFL. Routinely, four base-line TFL values were averaged and used as the predrug latency. The light source was set at an intensity that yielded baseline values of 2.5 to 3.5 sec. A 10-sec maximum exposure to the heat source was used to avoid damaging the tail tissue. Animals not responding within 10 sec were assigned a “cutoff” latency of 10. Data were expressed either as a percentage of the maximal possible effect where MPE = [(observed TFL − base-line TFL)/maximum TFL − base-line TFL] × 100 or as AUC, which was calculated as the change in postdrug TFL from the base-line latency plotted against time (ΔTFL × min) (see Tallarida and Murray, 1987, procedure 25, trapezoidal rule).

Because consistent responses to thermal stimuli applied to the tail are seen only if the base-line tail skin temperature remains constant (Berge et al., 1998), the tail and ambient room temperature were monitored throughout the experiments using an Omega DP 80 series Digital Indicator equipped with a 0.05-mm-diameter Copper Constantan Thermocouple.

**Data analysis.** When the statistical significance of the response to either neurotensin or morphine was to be assessed at various time intervals after injection, a one-way ANOVA with Fisher’s LSD post hoc test was used. A repeated-measures ANOVA with contrasts on dose was used when antinociceptive response to multiple doses of the agonists was evaluated at various time intervals. In experiments in which the magnitude of the change in the response to either morphine or neurotensin produced by various doses of SR 48692 was to be analyzed, a two-way ANOVA was used with contrasts on the difference between the response to the particular agonist given with various doses of SR 48692 (or vehicle) and the response (if any) to SR 48692 alone. A value of P < .05 was considered significant in all of these tests, and 6 rats were used in each study group. **JMP Statistics and Graphics Guide** version 3.1 (SAS Institute Inc., Cary, NC, 1995) was used as the resource text for the statistical analyses.

**Drugs.** Drugs used in these experiments were morphine (Mallinckrodt Chemical, St. Louis, MO); neurotensin (Sigma Chemical, St. Louis, MO); SR 48692, SR 48527 and SR 49711 (Sanofi Recherche, Toulouse Cedex, France) and levocabastine (Research Diagnostics, Flanders, NJ).

**Results**

**Influence of the initial microinjection of saline, DMSO, neurotensin or SR 48692 in the RVM on the tail-flick reflex and tail skin temperature.** An initial microinjection of saline, 100% DMSO (SR 48692 vehicle), SR 48692 or neurotensin caused an elevation in the temperature of the skin of the tail and a slight reduction in the TFL (table 1). The magnitude of the increase in temperature was variable, but the mean change ranged from ~1.5° to 4°C 10 min after the injections, and the corresponding decreases in TFL appeared to be inversely correlated with the increases. These changes, which lasted for ~15 min, were not observed when a second injection was performed 20 min after the first. A slight increase in tail temperature (mean value, 1.35°C) was also observed after the insertion of the injection cannula but without performance of a microinjection. Due to these alternations, which appear to be related to the initial mechanical disruption of the RVM tissue, responses in all subsequent experiments were quantified beginning at 20 min after the first injection of drugs or vehicles into the RVM.

**Influence of the microinjection SR 48692 in the RVM on the tail-flick reflex and behavioral responses.** When doses of SR 48692 of ≥0.3 pmol were injected into the RVM, a slight but significant elevation of the TFL occurred beginning 30 min after the injection (fig. 1, doses of 0.03 fmol to 3000 pmol were studied, but only the responses (TFL) to doses of ≥0.03 pmol are illustrated). The response was maximal with the lowest effective dose (0.3 pmol) of the neurotensin antagonist. Although all doses of SR 48692 initially elevated tail skin temperature (as above for 10–15 min; data

<table>
<thead>
<tr>
<th>Substance injected</th>
<th>Preinjection</th>
<th>First injection</th>
<th>Second injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/saline</td>
<td>22.77 ± 0.05</td>
<td>24.38 ± 0.25</td>
<td>22.77 ± 0.03</td>
</tr>
<tr>
<td>DMSO/DMSO</td>
<td>3.43 ± 0.15</td>
<td>3.02 ± 0.15</td>
<td>3.45 ± 0.24</td>
</tr>
<tr>
<td>SR 48692/saline</td>
<td>26.1 ± 0.16</td>
<td>28.97 ± 0.80</td>
<td>26.33 ± 0.20</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>3.32 ± 0.11</td>
<td>2.77 ± 0.25</td>
<td>3.37 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>22.90 ± 0.26</td>
<td>26.30 ± 0.75</td>
<td>23.78 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>3.23 ± 0.10</td>
<td>2.55 ± 0.22</td>
<td>3.18 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>24.63 ± 0.11</td>
<td>28.63 ± 1.25</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>3.18 ± 0.15</td>
<td>4.47 ± 0.90</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* SR 48692 dose was 0.03 pmol, a dose that did not subsequently cause an increase in TFL (fig. 1); the second injection was performed with saline.

* Neurotensin dose was 10 nmol, a dose that is antinociceptive, thus accounting for the rise in TFL, even though tail temperature is increased. A second injection was not administered in this experimental group (N.D.).
which they elevated these latency values. Served between the doses (matched-pairs respective predrug levels between 30 and 60 min after the injection nearly all of the latency values were significantly increased above their tially injected into the RVM, the antagonist allowed a dose-in some pain-inhibitory tone. Of a neurotensin seems to require that the animal be express-

Thus, the consistent expression of the pain-facilitatory effect in the tail-flick test is increased by stressing the rats. The antinociceptive response is illustrated as mean TFL. The S.E. was not included for the 0.3-pmol dose of SR 48692. Time is expressed in min after the injection of SR 48692. With doses of SR 48692 $\geq 0.3$ pmol, nearly all of the latency values were significantly increased above their respective predrug levels between 30 and 60 min after the injection (matched-pairs t test). In addition, no significant difference was observed between the doses $\geq 0.3$ pmol with respect to the degree to which they elevated these latency values.

not shown), the corresponding facilitation of the tail-flick response at 10 min after injection was not observed with doses of the antagonist that were $\geq 30$ pmol and were in the range that subsequently elevated the TFL (fig. 1).

To determine whether the microinjection of SR 48692 produced alterations in the behavior of rats, some of the animals were removed from their cages at various times after the injection. By gross observation, these rats did not appear to exhibit altered behavior; they were neither sedated nor show signs of motor dysfunction, such as ataxia.

**Influence of SR 48692 (RVM) on the tail-flick response to the RVM administration of a dose (30 pmol) of neurotensin associated with pain facilitation.** A dose of 30 pmol of neurotensin injected into the RVM has been previously described as producing a brief (i.e., 20–30 min) decrease in TFL in a significant proportion of rats (see Urban and Smith, 1993, fig. 2). However, the ability to resolve hyperalgesia in subsequent studies has been variable. In fact, in the current study (fig. 2A), the average response to a 30-pmol dose of neurotensin was a slight increase in TFL (illustrated as MPE, see Methods). The reason for this difference is unclear, but it may be partially related to the minimal sensitivity of the tail-flick test for demonstrating drug-induced decreases in the threshold of the tail-flick reaction (Hammond, 1989; Ness and Gebhart, 1986), coupled with the transient and slight nature of the action of low doses of exogenously administered neurotensin (Urban and Smith, 1993). On the other hand, it should also be noted that studies in progress demonstrate a consistent reduction in the TFL with this dose of neurotensin when the threshold to responding in the tail-flick test is increased by stressing the rats. Thus, the consistent expression of the pain-facilitatory effect of a neurotensin seems to require that the animal be expressing some pain-inhibitory tone.

When SR 48692 and neurotensin (30 pmol) were sequentially injected into the RVM, the antagonist allowed a dose-dependent expression of a marked antinociceptive response to the agonist (fig. 2A, repeated-measures ANOVA with contrasts). A significant antinociceptive response was observed beginning at a dose of 0.3 pmol of SR 48692. Doses of SR 48692 from 0.03 fmol to 3000 pmol, in 10-fold increments, were studied; however, for clarity, only the effects of selected doses are illustrated.

In figure 2B, data from the experiments represented by figure 1 (i.e., SR 48692 alone, ▲) and figure 2A (i.e., SR 48692 and neurotensin, ■) are expressed as AUC to provide a more clear representation of the dose-response relationship. These data confirm that the administration of doses of SR 48692 of $\geq 0.3$ pmol resulted in a response to neurotensin that was significantly greater than the minimal responses to neuro-
tensin alone and the various corresponding doses of SR 48692 (or vehicle) alone (two-way ANOVA with contrasts, see Methods). The potentiation of the response to neurotensin by SR 48692 was maximal and unchanging with doses between 3.0 and 3000 pmol.

**Influence of SR 48692 (RVM) on the tail-flick response to an antinociceptive dose (10 nmol) of neurotensin injected into the RVM.** A dose of 10 nmol of neurotensin injected into the RVM caused a significant increase in TFL (one-way ANOVA). The response, illustrated as MPE in figure 3A, peaked between 30 and 50 min (corresponding to 50–70 min after SR 48692 vehicle). Prior microinjection of various doses of SR 48692 resulted in a multiphasic alteration in the response to neurotensin (fig. 3, A and B; repeated-measures ANOVA with contrasts). The response of neurotensin was clearly attenuated (40–80 min after SR 48692) by doses of the antagonist from 0.1 to 3 fmol (fig. 3A), returned to values that were not significantly different from the control response between 30 fmol and 3 pmol (fig. 3, A and B) and were again significantly reduced (50–110 min after SR 48692), but not eliminated, at doses of >30 pmol (fig. 3B).

This pattern of the dose-dependent, multiphasic effect of SR 48692 on the response of neurotensin response is more clearly observed in figure 3C, where antinociception (calculated from the data in fig. 3, A and B) is illustrated as AUC. Partial antagonism of the response of neurotensin response is observed at both ends of the SR 48692 dose-response relationship. That is, response values obtained with neurotensin in combination with SR 48692 were significantly reduced but were still significantly greater than the values obtained with the corresponding doses of the antagonist alone (two-way ANOVA with contrasts). In contrast, the responses of neurotensin with intermediate doses of SR 48692 (30 fmol to 3 pmol) returned to values that were not significantly different from those observed at the dose levels (at either end of the multiphasic curve) where antagonism occurred.

A chimeric compound chemically similar to SR 48692 was used to determine whether the effect of high (>30 pmol) doses of the antagonist were due to stereospecific interactions at neurotensin receptors. The (S)-enantiomer SR 48527 ([S]-(+)-[1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]amino)cyclo-hexyl-acetic acid], has a high affinity ($K_i = 85$ nM) for neurotensin binding sites, whereas the (R)-enantiomer (SR 49711) exhibits limited affinity ($K_i = 6.2$ μM) (Labbe-Jullie et al., 1995). Because the binding affinity of the SR 48527 is ~5-fold less at neurotensin binding sites than is SR 48692, a dose of 300 pmol of each of the enantiomers was tested. At this dose level, only SR 48527 effectively reduced the antinociceptive effect of neurotensin (AUC: neurotensin/DMSO = 282 ± 70.3, neurotensin/SR 48527 = 75.7 ± 33.25, neurotensin/SR 49711 = 193 ± 57.38). Higher doses of the enantiomers were not used due to the limited supply available.

The influence of SR 48692 on the antinociceptive response to 10 nmol of neurotensin does not appear to be related to an interaction at low-affinity neurotensin binding sites that are sensitive to the histamine antagonist levocabastine (Schotte et al., 1986). The antinociceptive response to neurotensin was neither increased nor decreased by levocabastine used over a wide dose range (1 fmol to 1 nmol, RVM) (table 2). Although the response to neurotensin in the presence of 10 to 100 pmol of levocabastine appears to be increased over that observed...
with neurotensin alone, this increase in antinociceptive response is accounted for by an additivity of the response to neurotensin alone and the slight but significant antinociceptive effects of levocabastine at these doses (two-way ANOVA with contrasts). Work in progress is designed to determine whether this antinociceptive action of levocabastine is related to an action of the drug at neurotensin receptors or might be due to an action at histamine receptors.

Influence of SR 48692 (RVM) on the tail-flick response to an antinociceptive dose of morphine injected into the PAG or administered intraperitoneally. The microinjection of morphine (6 nmol) into the PAG resulted in an antinociceptive response that peaked in 30 to 40 min (i.e., 40–50 min after the injection of the vehicle for SR 48692 into the RVM; fig. 4A). The response returned to pre-drug levels within 120 min after the vehicle. Prior microinjection of various doses of SR 48692 into the RVM resulted in a bell-shaped enhancement of both the peak and duration of the response to morphine. The effective doses of SR 48692 were 3 to 300 pmol, and they enhanced the response of morphine between the time intervals of 30 to 120 min after the antagonist (fig. 4A; repeated-measures ANOVA with contrasts). However, the highest dose (3000 pmol) of SR 48692 was ineffective, with the response to morphine being no greater than that observed in the absence of the antagonist. By analyzing the AUC values from these data (fig. 4B; two-way ANOVA with contrasts), it was confirmed that the response of morphine was significantly enhanced by the effective doses of SR 48692 and that the degree of enhancement was not different within this dose range of the antagonist.

In contrast to this bell-shaped dose-response relationship for SR 48692 against PAG morphine, when morphine (2 mg/kg) was injected intraperitoneally, its antinociceptive response was only dose-dependently enhanced by SR 48692 injected into the RVM (fig. 5A and B). The response of morphine, which peaked within 30 min and returned to pre-drug levels within 120 min after SR 48692 vehicle, was enhanced in both magnitude and duration by doses of SR 48692 between 3 and 3000 pmol (fig. 5A; repeated measures ANOVA with contrasts demonstrated significant increases extending to 180 min after injection of SR 48692. Although significantly greater than the response to neurotensin and vehicle, the increase is accounted for by the added response to levocabastine and neurotensin alone (P > .05, two-way ANOVA with contrasts on the difference between neurotensin with levocabastine and the corresponding dose of levocabastine alone vs. the difference between neurotensin and levocabastine vehicle).

Table 2: Influence of levocabastine (RVM) on the antinociceptive response (AUC) to neurotensin (RVM)

<table>
<thead>
<tr>
<th>Levocabastine dose (pmol)</th>
<th>AUC Levocabastine</th>
<th>Levocabastine + 10 nmol of neurotensin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Vehicle)</td>
<td>−2.94 ± 2.32</td>
<td>149.12 ± 25.39</td>
</tr>
<tr>
<td>0.0001</td>
<td>26.54 ± 11.0</td>
<td>143.10 ± 25.93</td>
</tr>
<tr>
<td>0.001</td>
<td>−0.82 ± 3.55</td>
<td>173.48 ± 52.35</td>
</tr>
<tr>
<td>0.01</td>
<td>25.01 ± 6.01</td>
<td>137.84 ± 43.41</td>
</tr>
<tr>
<td>0.1</td>
<td>46.53 ± 16.48</td>
<td>149.28 ± 34.69</td>
</tr>
<tr>
<td>1.0</td>
<td>37.12 ± 13.46</td>
<td>219.24 ± 43.84</td>
</tr>
<tr>
<td>10.0</td>
<td>71.66 ± 16.39</td>
<td>230.96 ± 24.07</td>
</tr>
<tr>
<td>100.0</td>
<td>87.66 ± 28.22</td>
<td>246.23 ± 53.16</td>
</tr>
<tr>
<td>1000.0</td>
<td>12.12 ± 5.28</td>
<td>133.94 ± 21.2</td>
</tr>
</tbody>
</table>

* AUC quantified between 20 and 140 min after the injection of levocabastine. Levocabastine was administered first with the injection of neurotensin or saline 20 min later.

** Although significantly greater than the response to neurotensin and vehicle, this increase is accounted for by the added response to levocabastine and neurotensin alone (P > .05, two-way ANOVA with contrasts on the difference between neurotensin with levocabastine and the corresponding dose of levocabastine alone vs. the difference between neurotensin and levocabastine vehicle).

Influence of SR 48692 (RVM) on the tail-flick response to an antinociceptive dose of morphine injected into the PAG or administered intraperitoneally. The microinjection of morphine (6 nmol) into the PAG resulted in an antinociceptive response that peaked in 30 to 40 min (i.e., 40–50 min after the injection of the vehicle for SR 48692 into the RVM; fig. 4A). The response returned to pre-drug levels within 120 min after the vehicle. Prior microinjection of various doses of SR 48692 into the RVM resulted in a bell-shaped enhancement of both the peak and duration of the response to morphine. The effective doses of SR 48692 were 3 to 300 pmol, and they enhanced the response of morphine between the time intervals of 30 to 120 min after the antagonist (fig. 4A; repeated-measures ANOVA with contrasts). However, the highest dose (3000 pmol) of SR 48692 was ineffective, with the response to morphine being no greater than that observed in the absence of the antagonist. By analyzing the AUC values from these data (fig. 4B; two-way ANOVA with contrasts), it was confirmed that the response of morphine was significantly enhanced by the effective doses of SR 48692 and that the degree of enhancement was not different within this dose range of the antagonist.

In contrast to this bell-shaped dose-response relationship for SR 48692 against PAG morphine, when morphine (2 mg/kg) was injected intraperitoneally, its antinociceptive response was only dose-dependently enhanced by SR 48692 injected into the RVM (fig. 5A and B). The response of morphine, which peaked within 30 min and returned to pre-drug levels within 120 min after SR 48692 vehicle, was enhanced in both magnitude and duration by doses of SR 48692 between 3 and 3000 pmol (fig. 5A; repeated measures ANOVA with contrasts demonstrated significant increases extending to 180 min after injection of SR 48692. Although significantly greater than the response to neurotensin and vehicle, the increase is accounted for by the added response to levocabastine and neurotensin alone (P > .05, two-way ANOVA with contrasts on the difference between neurotensin with levocabastine and the corresponding dose of levocabastine alone vs. the difference between neurotensin and levocabastine vehicle).

Influence of the systemic administration of SR 48692 on the response to an antinociceptive dose of morphine administered either into the PAG or intraperitoneally. The systemic administration of SR 48692 alone resulted in an antinociceptive response only when administered in the highest dose tested (10 mg/kg i.p., data not shown). The response was small (~10% MPE) and was observed at 10 to 70 min after the injection. No change in tail skin temperature was seen after the systemic administration of any dose of the antagonist.

On the other hand, similar to its effect after RVM injection, the systemic administration of SR 48692 also resulted in a marked enhancement of the antinociceptive response to mor-
phine (6 nmol) injected into the PAG (fig. 6, A and B, which represents data from a narrower dose range from a separate study performed with doses in the effective range; table 3). The dose-response relationship was bell-shaped with the doses between 0.1 and 0.3 mg/kg (i.p.) enhancing the effect of morphine, whereas higher doses (1 mg/kg and above) and lower doses (<0.1 mg/kg) were ineffective (repeated-measures ANOVA with contrasts). However, none of the SR 48692 doses significantly reduced the response of morphine to values below that observed in the absence of the antagonist.

Similarly, SR 48692 (i.p.) caused a bell-shaped, dose-dependent enhancement of the response to an antinociceptive dose of morphine (2 mg/kg) administered intraperitoneally (fig. 7, A and B, which represents a narrower dose range; table 3). The effective doses of SR 48692 were 0.03 to 0.3 mg/kg and essentially in the same range as that observed after PAG administration of morphine.

Discussion

The results of the current study confirm that exogenously administered neurotensin dose-dependently activates opposing pain-facilitatory and -inhibitory neuronal processes in the RVM, with pain facilitation being the most prominent action of low (picomolar) doses of neurotensin, whereas antinociception is dominant at higher (nanomolar) doses. Furthermore, the pain-facilitatory component appears to reduce the antinociceptive potential of the exogenously administered neurotensin because dose-selective antagonism of neurotensin receptors by SR 48692 causes an otherwise ineffective dose of neurotensin (i.e., the dose promoting pain facilitation, see the introduction) to become distinctively antinociceptive.

The physiological significance of a bipolar action of neurotensin within the RVM is not fully understood. A number of studies have demonstrated inhibitory and facilitatory behavioral and neuronal responses from this area (Fields et al., 1991; Urban and Gebhart, 1994; Urban and Smith, 1993, 1994; Zhuo and Gebhart, 1990, 1992). However, in the current study, neurotensin receptor blockade in the absence of exogenously administered neurotensin results only in an increase in TFL. Thus, endogenous neurotensin mechanisms may function predominately, or exclusively, in a pain-facilitatory manner within the RVM. On the other hand, this result should be interpreted cautiously because others ob-
serve that the ability to distinguish bipolar effects of the neurotensin may depend on the extent to which animals are challenged with noxious stimuli. For example, Bodnar et al. (1982) demonstrated that when neurotensin antiserum is administered intracerebroventricularly, either hyperalgesia or antinociception is observed depending on the intensity of noxious stimulation that is applied. These data suggest that neurotensin neuronal circuitry maintains a homeostatic balance of pain responsivity in animals, and perhaps the neurotensin neuronal projections from the PAG to the RVM play a critical role in such homeostasis.

It is speculated on the basis of the current study that the dose-selective actions of SR 48692 and neurotensin on pain modulation in the RVM are explained by interactions with multiple neurotensin receptor subtypes. In this respect, two neurotensin receptors have been cloned, one of which is levocabastine sensitive with a low affinity for neurotensin (Chalon et al., 1996) and the other of which is insensitive to levocabastine and has high affinity for neurotensin (Tanaka et al., 1990; Vita et al., 1993). These receptors also exhibit differential affinity for SR 48692, with the high-affinity neurotensin site being ~10 times more sensitive to the antagonist (IC$_{50}$ values for competing with 125I-labeled neurotensin in rat brain tissue or COS-7 cells transfected with the cloned high-affinity rat brain receptor are 4.0 and 8.7 nM, respectively) than the low-affinity site (IC$_{50}$ value is ~80 nM in rat brain tissue) (Gully et al., 1993). The levocabastine-sensitive, low-affinity neurotensin receptors apparently are not involved in the actions of neurotensin in the RVM because the antinociceptive action of neurotensin was unaltered by levocabastine. Thus, the multiple receptors that appear to be involved in pain modulation from this brain site may tentatively be suggested to exist as subtypes within the class of binding sites expressing higher affinity for neurotensin. Subtypes within this class have been difficult to resolve neurochemically (Le et al., 1996; Vincent, 1995). However, it is generally conceded that they must exist to explain dose-selective actions of various neurotensinergic compounds on neurochemical and physiological processes (Gully et al., 1993; Labbe-Jullie et al., 1994; Le et al., 1996; Poncelet et al., 1994; Pugsley et al., 1994; Steinberg et al., 1994; Vincent, 1995). Of the multiple neurotensin receptors that appear to be resolved in this study, several seem to be associated with antinociceptive functions of neurotensin, whereas another is involved in its pain-facilitatory action. This is supported by the data demonstrating a triphasic antagonist dose-response relationship for the effect of SR 48692 against the selective antinociceptive dose (10 nmol) of neurotensin coupled with a comparative analysis of the doses of the antagonist required to block receptors modulating the predominate pain-facilitatory actions of the low dose (30 pmol) of neurotensin. For example, low (femtomolar) doses of the SR 48692 attenuate the antinociceptive response to neurotensin, whereas higher (picomolar) doses, presumably by blocking the pain-facilitatory component of the action of neurotensin, reversed the inhibition. The latter is assumed because the doses of SR 48692 associated with this reversal correspond to those that cause the ineffective dose of neurotensin (i.e., the dose pro-

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**TABLE 3**

<table>
<thead>
<tr>
<th>SR 48692 dose (mg/kg i.p.)</th>
<th>AUC</th>
<th>PAG morphine (6 nmol) + SR 48692 (i.p.)</th>
<th>Systemic morphine (2 mg/kg i.p.) + SR 48692 (i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Vehicle)</td>
<td>−2.94 ± 2.32</td>
<td>174.22 ± 17.10</td>
<td>108.10 ± 10.83</td>
</tr>
<tr>
<td>0.03</td>
<td>4.85 ± 2.89</td>
<td>237.79 ± 69.94</td>
<td>479.50 ± 97.25</td>
</tr>
<tr>
<td>0.1</td>
<td>2.06 ± 3.59</td>
<td>464.97 ± 101.88</td>
<td>501.33 ± 63.30</td>
</tr>
<tr>
<td>0.3</td>
<td>6.47 ± 2.77</td>
<td>695.17 ± 134.29</td>
<td>412.00 ± 84.97</td>
</tr>
</tbody>
</table>

* AUC (quantified between 30 min after the injection of SR 48692 and when the response to morphine had returned to within 10% of predrug levels from data in figs. 6B and 7B, and data not shown for SR 48692 alone).

* Significantly greater response of morphine (P < .05, two-way ANOVA with contrasts on the difference between morphine with various doses of SR 48692 and the corresponding doses of SR 48692 alone vs. the difference between morphine and the SR 48692 vehicle [Tween/saline, see Methods]).

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**Fig. 7.** Influence of SR 48692 (i.p.) on the response to an antinociceptive dose of morphine (2 mg/kg) injected intraperitoneally. A, Data from experiments in which SR 48692 was administered over a wide dose range. B, Data from experiments using a narrower dose range of SR 48692 within the effective range. In both A and B, SR 48692 was injected at time 0, and morphine was injected 30 min later, and the response is illustrated as MPE (see legend to fig. 2) ± S.E. All data points for the combination of morphine with doses of SR 48692 between 0.03 and 0.3 mg/kg i.p. were significantly different from the response to morphine alone beginning 50 min after SR 48692.
moting pain facilitation) to become clearly antinociceptive. Then, as the dose of SR 48692 was increased to higher levels (>30 pmol), the antinociceptive response to neurotensin was again depressed. The second inhibition was incomplete, allowing a significant proportion of the response of neurotensin to remain unchanged, even though the dose of SR 48692 was increased 100-fold (i.e., to 3000 pmol). Thus, a portion of the antinociceptive effect of neurotensin appears to be relatively insensitive to SR 48692. Therefore, based on the effective dose ranges of neurotensin and SR 48692 resolved in the current study, receptors with the following apparent affinities for the agonist and antagonist are proposed to exist within the RVM: a pain-facilitatory receptor with a high affinity for neurotensin and an intermediate affinity for SR 48692 and antinociceptive receptors with lower affinity for neurotensin that exhibit high, low or limited if any affinity for SR 48692, respectively.

It is important to note in this analysis that the effects of high doses of SR 48692 do not appear to be associated with a nonspecific or non-neurotensin receptor-mediated interaction. In fact, a high dose of the compound SR 48527 (which is chemically related to SR 48692) administered in a dose that should interact in a kinetically equivalent manner with neurotensin receptors (Labbe-Jullie et al., 1995) produced a similar inhibition of the antinociceptive response to neurotensin, whereas the same dose of the inactive enantiomer (SR 49711) of the analog was found to be ineffective. Furthermore, it is unlikely that the actions of high doses of the SR 48692 are a consequence of diffusion to distant neurotensin receptors involved in antinociception because it has been previously shown that the area of the RVM that was the target microinjection site in these studies (i.e., RMg) is the most prominent site within this brain area for evoking the antinociceptive effect of neurotensin (Urban and Smith, 1994).

In an earlier study performed by Dubuc et al. (1994), it was suggested that SR 48692 was ineffective as an antagonist of the antinociceptive effect of neurotensin. They demonstrated that SR 48692 administered systemically (i.p. or p.o.) did not interfere with the antinociceptive effect of intracerebroventricularly administered neurotensin in the writhing and tail-flick tests performed in mice and rats. Although these results are consistent with the idea that some neurotensin receptors are insensitive to SR 48692, it is unclear given the results of the present study why these investigators did not resolve at least a partial antagonist-induced attenuation of the neurotensin response. However, these studies differ in the distribution of effective concentrations of neurotensin and SR 48692 to various neurotensin neuronal circuitry involved in pain modulation (Al-Rodhan et al., 1991; Behbehani, 1992; Clineschmidt et al., 1979; Yaksh et al., 1982). The approach used by Dubuc et al. (1994) exposes a variety of supraspinal brain areas to neurotensin (intracerebroventricularly) and the entire CNS to SR 48692 (systemic administration), whereas in the current study the effects of these drugs should be restricted to the RVM. Moreover, the concentration of neurotensin diffusing to the RVM from the ventricular space in the Dubuc study is likely to be lower that achieved after the direct microinjections in the site. Accordingly, they may have achieved a concentration that has limited ability to promote antinociception from the RVM where SR 48692-sensitive, neurotensin receptors clearly exist.

With respect to the pharmacological significance of neurotensin neurons projecting from the PAG to the RVM in the action of opioids, it appears that neurotensin mechanisms subserving pain-facilitatory functions are most important. The data suggest that a significant population of neurotensin neurons activated by the PAG administration of morphine function to oppose the antinociceptive action of the opioid and should be classified as functionally antianalgesic (Maier et al., 1992). Moreover, the demonstration that antinociceptive response to systemically administered morphine was also greatly enhanced by microinjecting SR 48692 into the RVM confirms the pharmacological significance of this opioid-activated, pain-facilitatory neurotensin relay.

On the other hand, the role of the antinociceptive actions of neurotensin in the pharmacology of opioids is less apparent. SR 48692 administered either systemically or directly into the RVM only enhanced the action of morphine, regardless of whether the opioid was given systemically or directly into the PAG. Generally, the enhancement was biphasic with higher doses of the antagonist appearing to lose their ability to potentiate the action of the opioid. In fact, it is important to note that there was no instance in which SR 48692 lowered opioid-induced antinociception to levels below that observed in control rats. Thus, the biphasic antagonist dose-response curves do not appear to occur as a consequence of higher doses of SR 48692 blocking an opioid-mediated antinociceptive mechanism dependent on neurotensin. Taken together, these data suggest that a morphine antinoiception may not be dependent on the specific neurotensin neuronal processes at any level of the CNS at which neurotensin has been implicated as an antinociceptive neurotransmitter (e.g., PAG: Al-Rodhan et al., 1991; Behbehani, 1992; intracerebroventricularly: Clineschmidt et al., 1979; spinal cord: Yaksh et al., 1982), including the specific PAG-to-RVM circuitry (Behbehani, 1992; Fang et al., 1987).

The reason for a bell-shaped dose-response relationship observed for the action of SR 48692 against the antinociceptive effect of morphine, as well as neurotensin (see above), is not understood. However, the appearance of multiphasic dose-response curves is a characteristic of the action of the antagonist on other physiological processes as well (e.g., inhibition of neurotensin-induced turnover behavior; Ponecet et al., 1994). In these studies, it was determined that the reversal of effectiveness of SR 48692 and other similar neurotensin receptor antagonists (e.g., SR 142948A) is blocked by pretreatment of animals with dopaminergic receptor antagonists (Gully et al., 1997). In this respect, because dopamine is a transmitter involved in pain modulation (particularly within the RVM; Phillips et al., 1986), SR 48692-sensitive neurotensin receptors associated with dopaminergic synapses (see Kitabgi, 1989) may provide the substrate for the biphasic action of the antagonist in antinociceptive mechanisms. Additional studies are in progress to evaluate this possibility.

In conclusion, these results confirm that neurotensin has dose-dependent, presumably receptor-selective, actions on pain modulation (Urban and Smith, 1993) and demonstrate that SR 48692 is capable of dose-dependently modulating the antinociceptive response to either neurotensin or morphine. In fact, neurotensin should now clearly join the growing number of neuropeptides (i.e., neuropeptide Y, cholecystokinin, neuropeptide FF and others; see review by Maier et al., 1992) that are distinguished by their dose-dependent bipolar
pain-facilitatory and antinoceptive actions (Oberling et al., 1993; Pittaway et al., 1987; Xu et al., 1994). Moreover, the development of receptor antagonists that are selective for the pain-facilitatory role expressed by neurotensin and/or these other peptides may make it possible to minimize opioid toxicity or overcome opioid tolerance when the antagonists are administered concomitantly with opioids.

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