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The NH_2-Terminus of Substance P Modulates NMDA-induced Activity in the Mouse Spinal Cord

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Excitatory amino acids (EAAs) and substance P are believed to transmit nociceptive information in the spinal cord. As substance P NH_2-terminal fragments can modulate non-NMDA EAA-mediated activity, we examined the effects of substance P fragments to ascertain whether the COOH- or NH_2-terminus of substance P modulates the actions of NMDA in the spinal cord. NMDA activity was measured by the intensity of behaviors produced by NMDA (0.2 nmol) administered intrathecally in the mouse. The NMDA response was attenuated after pretreatment with either substance P (22.5 pmol, 30 min) or the NH_2-terminal fragment of substance P, SP-(1-7). Pretreatment with the COOH-terminal fragment SP-(5-11) (22.5 pmol, 30 min), a neurokinin ligand, had no effect on NMDA-induced behaviors, suggesting that the inhibitory effect of substance P is caused by the NH_2-terminus. Pretreatment with D-Pro^2,D-Phe^7 substance P-(1-7), a SP-(1-7) antagonist, potentiated NMDA activity, suggesting a tonic inhibitory effect of the substance P NH_2-terminus. Desensitization to NMDA typically develops when NMDA is injected at 2 min intervals. While pretreatment with SP-(1-7) inhibited NMDA, coadministration of SP-(1-7) (22.5 pmol), with the first of four injections of NMDA, first inhibited but then potentiated responses to each challenge with NMDA. Coadministration of the same dose of SP-(1-7) with the fourth injection of NMDA immediately potentiated the response to NMDA. D-Pro^2,D-Phe^7 substance P-(1-7) blocked the inhibition but not the potentiation of NMDA by SP-(1-7) whereas DPDT-SP, a neurokinin receptor antagonist, failed to alter either effect of SP-(1-7). These data support the novel hypothesis that substance P inhibits phasic but potentiates tonic NMDA activity in the spinal cord by an action of its NH_2-terminus on two distinct non-neurokinin receptor populations.

[Key words: substance P, NMDA, spinal cord, nociception, modulation, mouse]

A growing body of data implicate excitatory amino acids (EAAs) and the neuropeptide substance P in the transmission of nociceptive information in the dorsal horn of the spinal cord (Salt and Hill, 1983; Besson and Chaouch, 1987; Schneider and Perl, 1988). The actions of EAAs and substance P appear to be closely interrelated, as both are found in high concentrations in the dorsal horn, where they are known to be colocalized in primary afferent C-fiber terminals (De Biasi and Rustioni, 1988; Tracey et al., 1991) and released in response to pain.

Interactions between substance P and EAAs have previously been shown to occur. Substance P causes the release of EAAs in the dorsal spinal cord (Smullen et al., 1990) and potentiates both the NMDA-induced cell firing of spinothalamic neurons and the depolarizations caused by noxious mechanical stimulation when substance P is applied iontophoretically (Dougherty and Willis, 1991) or released via peripheral application of capsaicin (Dougherty and Willis, 1992). These effects persist for more than 2 hr. Substance P also potentiates the behavioral effects of NMDA when coinjected in the mouse spinal cord (Mjellem-Joly et al., 1991).

Blockade of substance P activity at neurokinin receptors (Ohkubo et al., 1990) or EAA activity at either NMDA or non-NMDA receptors (Cahusac et al., 1984; Coderre and Melzack, 1991; Davar et al., 1991; Näsström et al., 1992) causes antinociception while application of substance P (Matsumura et al., 1985) and NMDA (Aanonsen and Wilcox, 1987) results in hyperalgesia. In contrast, most types of hyperalgesia are attenuated by pretreatment with capsaicin (Shir and Seltzer, 1990), which produces a long-term decrease in the release of neurotransmitters, including substance P, from primary afferent C-fibers (Buck and Burks, 1986). Treatment with MK-801, a PCP ligand that noncompetitively inhibits NMDA activity, is also effective in preventing hyperalgesia (Davar et al., 1991).

The activity of substance P and EAA agonists in the spinal cord at neurokinin and EAA receptors, respectively, can be monitored using a behavioral paradigm. Administration of EAA agonists (Hylden and Wilcox, 1983; Aanonsen and Wilcox, 1986) or substance P (Hylden and Wilcox, 1981) by the intrathecal route in mice produces a stereotypical biting and scratching behavior that persists for 1–2 min. While it remains unclear whether this effect reflects pain, this assay is an easily quantifiable and reproducible measure of activity produced by receptor populations believed to be involved in nociception. Thus, this assay allows us to study the susceptibility of activity produced by these receptors to manipulation by compounds involved in nociceptive processing.

Substance P has been reported to produce nociceptive as well as antinociceptive effects, depending on the dose and time of administration (Frederickson et al., 1978). Substance P–induced pain is thought to result from an interaction of its COOH-terminus with neurokinin receptors while the antinociceptive effect has been proposed to result from the release of endogenous opioids, based on its sensitivity to opioid antagonists (Frederickson et al., 1978). However, the hyperalgesic effect of substance P is not always blocked by neurokinin antagonists (Mat-

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sumura et al., 1985), suggesting that mechanisms other than neurokinin receptor activation may play a role in substance P–induced activity. It is, therefore, of interest that an NH_2-terminal metabolite of substance P, SP-(1-7), has also been shown to be antinociceptive in several assays (Stewart et al., 1982; Goettl and Larson, 1991) as well as hyperalgesic (Cridland and Henry, 1988). SP-(1-7) has no sequence homology with tachykinins and NH_2-terminal fragments do not appear to compete for binding at neurokinin receptors (Hanley et al., 1981). However, like substance P, the antinociceptive effect of SP-(1-7) is reversed by naloxone, suggesting a common mechanism (Goettl and Larson, 1991). The hyperalgesic effect of SP-(1-7) is blocked by MK-801, implicating an activation of pathways containing NMDA-type receptors. Based on the previously described ability of substance P and SP-(1-7) to potentiate electrophysiologic responses to NMDA, we hypothesized that the NH_2-, rather than the COOH-terminus of substance P, together perhaps with an accumulation of substance P NH_2-terminal metabolites, plays a role in the hyperalgesic effect of substance P by an ability of the NH_2-terminal sequence to facilitate NMDA-induced activity.

Tonic exposure of the spinal cord to NMDA alone typically leads to desensitization (Sun and Larson, 1991). While the mechanism underlying desensitization to the behavioral effects of NMDA remains unknown, SP-(1-7) appears to affect NMDA-induced activity as SP-(1-7), applied iontophoretically to nociceptive-sensitive neurons in the rat spinal cord, produces an initial inhibition followed by potentiation of NMDA-induced activity (Budai et al., 1992). The potentiative effect of substance P on NMDA, originally observed by Dougherty and Willis (1991), may thus be due to an action of the NH_2- rather than its COOH-terminus of substance P. The present investigation was designed to test the hypothesis that the NH_2-terminus of substance P is capable of modulating NMDA-induced activity in the spinal cord of conscious, intact animals. We examined the effect of SP-(1-7) on NMDA-induced behavior after single as well as repeated injections of NMDA to compare the modulatory action of SP-(1-7) on acute and tonic EAA activity.

Materials and Methods

Male Swiss-Webster mice, 20–25 gm (Biolab, White Bear Lake, MN; Sasco, Omaha, NE) were housed four per cage and allowed to acclimate for at least 24 hr prior to experiments. Mice were allowed free access to food and water. Animals were used strictly in accordance with the guidelines of the University of Minnesota Animal Care and Use Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW publication (NIH) 78-23, revised 1978].

All injections were made intathecally at approximately the L5–L6 intervertebral space using a 0.5 inch, 30 gauge disposable needle mounted on a 50 μl Luer-tip syringe (Hamilton Co, Reno, NV). A cannula, constructed of a 30 gauge needle attached to PE-10 tubing mounted on a 50 μl syringe, was used for repeated injections as described previously (Larson and Beitz, 1988). For all injections, drugs were delivered in 5 μl volumes.

Immediately after insertion of the cannula, animals were placed in a large glass cylinder containing 2 cm of bedding. One minute later, mice were injected with 0.2 nmol of NMDA and the total number of caudally directed biting and scratching behaviors occurring over the subsequent 2 min interval was counted and expressed throughout as the response occurring at the 0–2 min interval. Each bite or scratch was counted as a single behavior. During episodes of persistent biting or scratching behavior, each second of continuous biting or scratching was counted as a single behavior. Mice were then reinjected with 0.2 nmol of NMDA. Behaviors were similarly counted for a total of four injections. The average number of responses elicited by the first, second, third, and fourth injections were expressed graphically at the 0–2, 2–4, 4–6, and 6–8 min intervals, respectively. The dose of NMDA used was chosen as it reliably produced the same number of biting and scratching behaviors and was of a magnitude that readily permitted measurement of inhibition or potentiation of behaviors. Repeated injections of vehicle failed to produce biting and scratching behaviors and had no effect on the animals' normal exploratory behavior. Pretreatment with vehicle produced no effect on subsequent NMDA-induced behaviors.

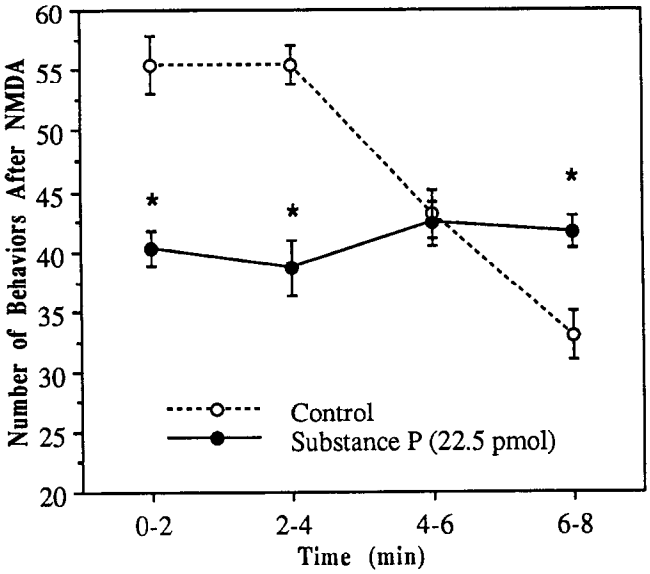

Figure 1. The inhibitory effect of substance P on the behavioral responses to NMDA. When NMDA (0.2 nmol) was injected in mice four times, every 2 min, there was a significant decrease in the intensity of caudally directed biting and scratching behaviors in control animals. When animals were pretreated with substance P (22.5 pmol) 30 min prior to the first injection of NMDA, the number of NMDA-induced behaviors in response to the first two injections is attenuated and no desensitization to the behavioral effect of NMDA occurs. All drugs were administered intrathecally. Each point represents six animals ±SEM. *Asterisks* indicate $p < 0.05$ compared to NMDA alone.

NMDA and D-Pro^2,D-Trp^{7,9} substance P were purchased from Sigma Chemical Co. (St. Louis, MO); substance P, substance P-(1-7), and substance P-(5-11) were purchased from Peninsula Laboratories (Belmont, CA); D-Pro^2,D-Phe^7 substance P-(1-7), a substance P NH_2-terminal antagonist, was synthesized by the University of Minnesota Microchemical Facilities (Minneapolis, MN). All drugs were dissolved in 0.85% saline containing 0.01 *N* acetic acid (Hall and Stewart, 1984).

Statistical analysis of data was performed using Student's *t* test for unpaired data for single comparisons. Multiple comparisons were performed using analysis of variance followed by Dunnett's test. In all cases, a level of significance was established at $p < 0.05$.

Results

Mice were initially pretreated with substance P (22.5 pmol) 30 min prior to the first of four injections of NMDA (0.2 nmol). This dose of substance P, which was chosen based on its ability to potentiate kainic acid–induced behaviors in mice (Sun and Larson, 1991), produced caudally directed biting and scratching behaviors that persisted for a period of less than 90 sec. Pretreatment with substance P attenuated the intensity of biting and scratching behaviors produced by NMDA compared to vehicle-pretreated groups and prevented the development of behavioral desensitization typically observed after four injections of NMDA (Fig. 1).

To determine whether the COOH- or NH_2-terminus of substance P was responsible for this inhibitory effect, mice were pretreated with SP-(1-7) (22.5 pmol) or SP-(5-11) (22.5 pmol)

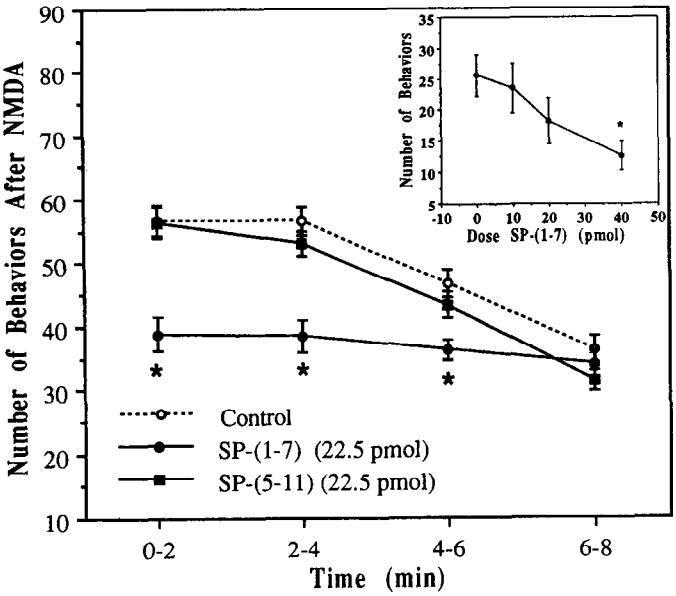

Figure 2. An NH_2-terminal but not a COOH-terminal fragment of substance P inhibits the behavioral response to NMDA. When the NH_2-terminal substance P metabolite, SP-(1-7) (22.5 pmol), was administered 30 min prior to a series of four injections of NMDA (0.2 nmol), there was a significant inhibition of NMDA-induced behaviors. In contrast, the COOH-terminal fragment SP-(5-11) (22.5 pmol) failed to elicit any change in NMDA-induced activity when similarly administered. *Inset*, The behavioral response of a single injection of NMDA is inhibited by pretreatment (30 min) with SP-(1-7) in a dose-dependent manner. Details are as in Figure 1.

30 min prior to the first of four injections of NMDA. Pretreatment with SP-(5-11), which itself produced a transient behavioral response, had no effect on subsequent NMDA-induced activity (Fig. 2). Similar to substance P, SP-(1-7) significantly inhibited NMDA-induced behaviors 30 min after its injection (Fig. 2) in a dose-related manner (Fig. 2, inset).

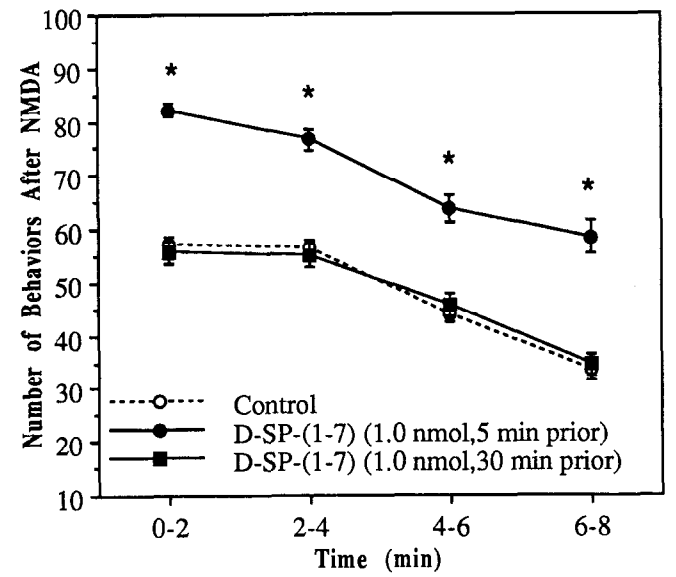

Figure 3. Potentiation of NMDA-induced behaviors by D-SP-(1-7). The SP-(1-7) antagonist D-SP-(1-7) (1.0 nmol) potentiated NMDA-induced behaviors when administered 5 min prior to the first of four injections of NMDA (0.2 nmol) but not when administered 30 min prior to NMDA. Details are as in Figure 1.

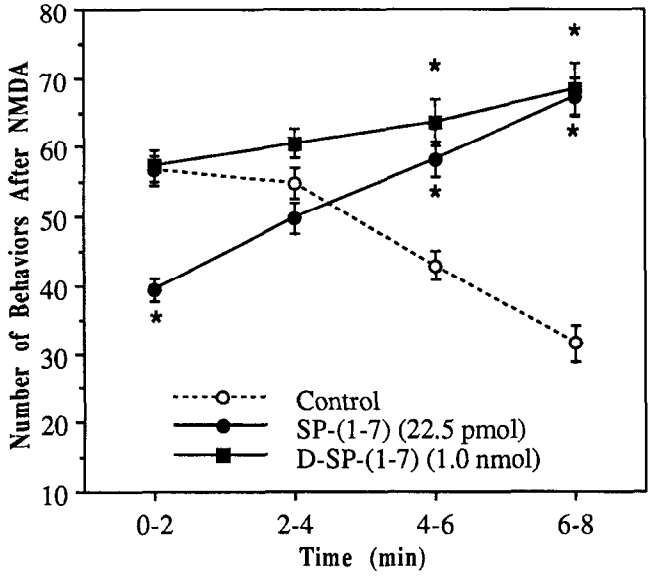

Figure 4. Coadministration of SP-(1-7) with NMDA. When SP-(1-7) (22.5 pmol) was coadministered with the first in a series of four injections of NMDA (0.2 nmol), there was a transient inhibition followed by a potentiation of NMDA-induced behaviors. When animals were pretreated with D-SP-(1-7) (1.0 nmol) 30 min prior, the inhibitory but not the excitatory effect of SP-(1-7) was blocked. Details are as in Figure 1.

The role of SP-(1-7) was further characterized using the SP-(1-7) antagonist D-SP-(1-7) at a dose that has been found to inhibit the potentiative effect of SP-(1-7) on kainic acid (Larson and Sun, 1992) as well as block the inhibitory effect of SP-(1-7) on substance P (Mousseau et al., 1992a). Injection of D-SP-(1-7) (1 nmol) 5 min prior to four intrathecal injections of NMDA resulted in a potentiation of the intensity of NMDA-induced behaviors with no change in the rate of desensitization (Fig. 3), suggesting that the NH_2-terminus of substance P is responsible for a tonic inhibitory action on NMDA receptor activity. Recovery from the potentiative effect of D-SP-(1-7) was complete by 30 min.

The influence of the NH_2-terminus of substance P on the response to NMDA was then examined at various times relative to the first injections of NMDA. SP-(1-7) (22.5 pmol) was coadministered with the first of four injections of NMDA, rather than as a 30 min pretreatment, resulting in an initial inhibition of behaviors induced by the first injection of NMDA, similar to the effect produced by pretreatment with SP-(1-7). The inhibition resulting from coadministered SP-(1-7) was transient, however, and immediately followed by a progressive increase in the intensity of behavioral responses to subsequent injections of NMDA (Fig. 4).

Whereas pretreatment with D-SP-(1-7) (1.0 nmol) 30 min prior to four injections of NMDA had no effect on behaviors elicited by NMDA (Fig. 3), pretreatment with D-SP-(1-7) blocked the inhibitory effect of SP-(1-7) without affecting the subsequent facilitation of NMDA-induced behaviors in animals in which SP-(1-7) (22.5 pmol) was coadministered with the first injection of NMDA (Fig. 4). In contrast, similar pretreatment with an equimolar injection of the neurokinin antagonist DPDT-SP (1.0 nmol) failed to alter the effects of coadministered SP-(1-7) on NMDA (Fig. 5) in spite of the ability of this dose of DPDT-SP to inhibit substance P-induced behaviors presumed to be induced by an interaction with neurokinin receptors.

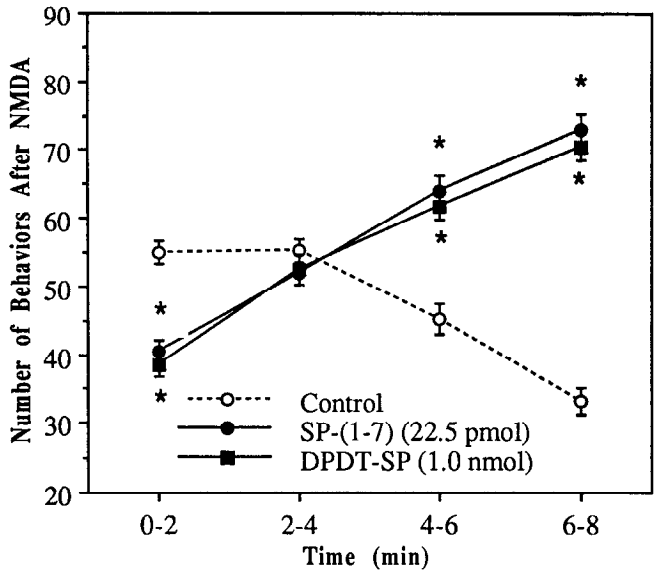

Figure 5. DPDT-SP, a neurokinin receptor antagonist, failed to alter the effects of SP-(1-7) on NMDA activity. Animals were pretreated with DPDT-SP (1.0 nmol) 30 min prior to a series of four injections of NMDA (0.2 nmol) in which SP-(1-7) (22.5 pmol) was coadministered in the first injection. Neither the inhibitory nor the excitatory effects of SP-(1-7) were altered. Details areas in Figure 1.

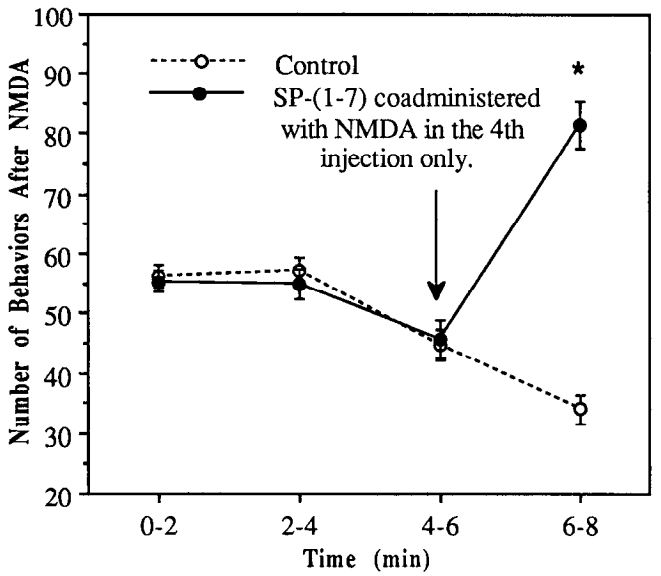

Figure 6. Coadministration of SP-(1-7) with the 4th injection of NMDA. In contrast to the effect of SP-(1-7) coadministered in the first injection of NMDA (Fig. 4), animals receiving SP-(1-7) (22.5 pmol) in the fourth injection of NMDA (as indicated by the *arrow*) displayed a marked potentiation in behavioral response. Details are as in Figure 1.

The effect of SP-(1-7) on NMDA activity appears to depend on the time of injection with respect to the four challenges with NMDA. In contrast to the effect of SP-(1-7) when injected with the first dose of NMDA, coadministration of SP-(1-7) (22.5 pmol) with the fourth injection of NMDA not only failed to inhibit, but produced an immediate and potent facilitation of NMDA-induced behaviors (Fig. 6), suggesting that the influence of substance P is dependent on the degree of NMDA-induced activity in the spinal cord at the time of substance P injection rather than the time interval between injections of substance P and NMDA.

Discussion

It has been suggested that substance P, acting as a putative transmitter of nociceptive information (De Koninck et al., 1992; Haley and Wilcox, 1992), may modulate NMDA activity along nociceptive pathways in the spinal cord (Dougherty and Willis, 1991, 1992). The present data illustrate a modulatory action of substance P on NMDA-induced behavioral activity in the spinal cord that occurs by an action of the NH_2- rather than the COOH-terminus of the substance P molecule. While the NH_2-terminal metabolites of substance P have been largely viewed as inactive by-products of the parent compound, our recent studies indicating that SP-(1-7) modulates neurokinin-induced and kainic acid–induced activity in the mouse spinal cord (Igwe et al., 1990a,b; Mousseau et al., 1992a; Sun and Larson, 1992), together with earlier work by Stewart and Hall (1982), support the concept that substance P modulates the activity of neurotransmitters by an action of its NH_2-terminus.

In contrast to the potentiative effects of substance P and SP-(1-7) on kainic acid-induced behaviors (Larson and Sun, 1992), pretreatment with substance P, 30 min prior to the first injection of NMDA, attenuated NMDA-induced behaviors (Fig. 1). This effect is brought about by the NH_2- rather than the COOH-terminus of substance P as pretreatment with SP-(1-7), which did not produce any behavioral response when injected alone, also inhibited NMDA-induced activity (Fig. 2) while SP-(5-11), which contains the same COOH-terminal sequence homology of other tachykinins, and interacts well with the neurokinin receptor, produced no effect when administered in an identical manner (Fig. 2).

D-SP-(1-7), which inhibits SP-(1-7) binding in mouse brain and spinal cord tissue homogenates (Igwe et al., 1990c), prevents the inhibitory effect of SP-(1-7) on substance P–induced behaviors (Mousseau et al., 1992a), and blocks the potentiative effect of SP-(1-7) on kainic acid–induced activity. In the present study, D-SP-(1-7) was also able to prevent the inhibitory, but not the potentiative, effect of SP-(1-7) coadministered with NMDA (Fig. 4). The selective attenuation of the inhibitory effect of SP-(1-7) by D-SP-(1-7) supports a role for the NH_2-terminus of substance P in the modulation of NMDA activity and suggests that the two opposing actions of SP-(1-7) on NMDA are brought about by two distinct receptors. Consistent with the conclusion that the NH_2-terminus of substance P inhibits NMDA, D-SP-(1-7) was able to potentiate NMDA-induced activity when administered immediately (5 min) prior to NMDA (Fig. 3). Together, these data suggest a possible tonic inhibitory influence of substance P or substance P NH_2-terminal metabolites on NMDA activity.

It is not likely that effects produced by SP-(1-7) are mediated via neurokinin receptors. SP-(1-7) contains no COOH-terminal sequence homology with tachykinins and NH_2-terminal metabolites of substance P do not interact with neurokinin binding sites (Hanley et al., 1981). Consistent with this, DPDT-SP, a neurokinin antagonist, was unable to inhibit the effect of SP-(1-7) in the present study (Fig. 5). That the neurokinin antagonist DPDT-SP is also unable to inhibit the mechanical hyperalgesia induced by substance P in mice (Matsumura et al., 1985) raises the possibility that a component of the hyperalgesic effect of substance P may also be brought about by an action of the NH_2-terminus of substance P.

The effect of SP-(1-7) on NMDA-induced activity appears to depend on the time of injection with respect to NMDA, producing an inhibitory response when administered prior to NMDA activity (Figs. 2, 4), but a pure excitatory effect when injected during a series of NMDA injections (Fig. 6). These data are consistent with our previous observations of the effect of SP-(1-7) microiontophoretically applied to nociceptive-sensitive dorsal horn neurons in the anesthetized rat in which SP-(1-7) also produced an initial inhibition followed by potentiation of repeated episodes of NMDA-induced depolarizations (Budai et al., 1992). One might speculate that the effect of SP-(1-7) depends on the activation state of NMDA receptor populations. The NMDA receptor is thought to exist as an elaborate receptor complex, controlled by a variety of different substances acting at modulation sites adjacent to the NMDA binding site. Agents, such as (±)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801, dizocilpine), noncompetitively antagonize NMDA receptor activity by binding allosterically at a site that has been proposed to be located within the activated ion channel (Kemp et al., 1987). Similarly, divalent cations, such as Mg^{2+} (Nowak et al., 1984) and Zn^{2+} (Westbrook and Mayer, 1987), each inhibit NMDA activity by binding to ion channel–associated sites. Several other modulators of NMDA activity have also been proposed, including glycine (Johnson and Ascher, 1987), polyamines (Ransom and Stec, 1988), and σ-receptor ligands (Monnet et al., 1992).

Activation of the NMDA receptor complex has been previously shown to result in complex allosteric changes, resulting in dramatic changes in ligand binding affinities (Javitt and Zukin, 1989a,b). For example, MK-801 binding is enhanced when the NMDA receptor is activated and decreased in the presence of competitive NMDA antagonists (Loo et al., 1986). The binding of ^{3}H-TCP, a PCP ligand, has been shown to be altered by substance P in rat brain (Jaffe et al., 1990) and by SP-(1-7) in mouse brain and spinal cord (Sun et al., 1992), suggesting a direct interaction of the substance P NH_2-terminus with the PCP receptor. These binding studies are consistent with the possibility that the modulatory effect of SP-(1-7) on NMDA-induced behaviors depends on the activation state of the NMDA receptor, similar to the dependence of PCP ligand binding on NMDA-induced activity. With respect to pain, such an arrangement would be expected to protect against the onset of NMDA-mediated pain signaling but, once activated, potentiate the intensity of NMDA receptor-mediated components of nociception.

The σ-receptor has also been proposed to be a multisite system that acts as a modulator of the NMDA receptor complex (Quirion et al., 1992). Ligands for the σ-receptor are also capable of modulating NMDA-induced excitatory activity in a dual fashion (Monnet et al., 1990, 1992), perhaps reflecting actions at two different subtypes of this receptor. While the endogenous ligand for the σ-receptor is unknown at this time, it has been proposed to be a peptide (Contreras et al., 1987). Substance P and SP-(1-7) each mimic the potentiative effect of 1,3-di-orthotolyl-guanidine (DTG), proposed to be a σ-receptor agonist (Monnet et al., 1990), whereas haloperidol acts as a σ-receptor antagonist (Monnet et al., 1990), preventing both DTG-induced and SP-(1-7)–induced actions on kainic acid-induced activity in the mouse spinal cord (Larson and Sun, 1993). This suggests that the NH_2-terminus of substance P may either interact with sigma sites, which occurs only at relatively high concentrations (Mousseau et al., 1992b) or evoke the release of an endogenous σ-ligand. One might speculate that SP-(1-7) produces a similar action, modulating acute and tonically evoked NMDA activity. These possibilities require further study.

In summary, our data indicate that substance P modulates NMDA-induced activity, resulting in a tonic inhibition of NMDA. While inhibition of NMDA receptor activity appears to be the dominant effect of SP-(1-7) in the absence of exogenously administered NMDA receptor agonist, SP-(1-7) potentiates tonic, or ongoing NMDA-induced activity. Both the inhibitory as well as the potentiative effects of SP-(1-7) appear to occur via an interaction of the NH_2-terminus of substance P with novel, non-neurokinin receptors.

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