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Tachykinin NK_1 receptor antagonists enhance stress-induced c-*fos* in rat locus coeruleus

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Abstract

These experiments tested the hypothesis that substance P neurotransmission at tachykinin NK_1 receptors in the locus coeruleus is involved in stress-induced activation of the locus coeruleus, using c-*fos* as an index of activation. Selective tachykinin NK_1 receptor antagonists administered systemically did not result in substantial locus coeruleus c-*fos* expression. Restraint stress resulted in a large number of locus coeruleus c-*fos* expressing cells. Administration of two selective tachykinin NK_1 receptor antagonists prior to restraint resulted in an increase in the number of locus coeruleus c-*fos* expressing cells, compared to restraint alone. These results suggest that the enhanced c-*fos* expression observed in response to tachykinin NK_1 receptor antagonists combined with stress, could be due to the blockade of tachykinin NK_1 receptor-mediated activity at sites other than the locus coeruleus, resulting in an overall activation of the locus coeruleus. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Locus coeruleus; Tachykinin NK_1 receptor; Neurokinin receptor; Substance P; c-*fos*; Stress

1. Introduction

The locus coeruleus noradrenergic system is responsive to many stimuli. External sensory events involving vigilance or orientation towards stimuli increase locus coeruleus firing rate in rats and monkeys (Aston-Jones et al., 1991). Physiological changes in the internal environment, such as hypotension and bladder distention, also increase the firing rate of locus coeruleus neurons in rats (Page and Valentino, 1994). Noxious stimuli such as a pinch or restraint increase locus coeruleus neuronal activity in animals (Abercrombie and Jacobs, 1987; Rasmussen et al., 1986). The robust response of locus coeruleus neurons in animals to noxious events suggests the possible involvement of the locus coeruleus in human psychiatric disorders involving anxiety and stress (Redmond, 1987).

Tachykinins mediate many effects, including relay of information in sensory neurons to the spinal cord, neurogenic inflammation, and vasoconstriction of smooth muscle (Otsuka and Yoshioka, 1993). Substance P, along with neurokinin A and neurokinin B, represent the tachykinins that are expressed in the mammalian central nervous system (Helke et al., 1990). There is evidence for a role for substance P in the central response to stress. Central administration of substance P produces a constellation of behaviors and cardiovascular effects mediated by the sympathetic nervous system that closely resemble the defense reaction and response to stress (Culman et al., 1995). Stressors such as restraint and footshock result in decreases in substance P levels in discrete brain regions, suggesting an increase in release of substance P under stress (Siegel et al., 1987; Takayama et al., 1986), and an antibody to substance P prevents a footshock-induced increase in mesocortical dopamine turnover (Bannon et al., 1983).

Studies demonstrate that the locus coeruleus contains tachykinin NK_1 receptors, the receptor selective for substance P. Radioligand binding demonstrates substance P binding in the locus coeruleus (Buck et al., 1986; Mantyh et al., 1989; Saffroy et al., 1988; Dam et al., 1990). In situ hybridization using probes directed against the tachykinin NK_1 receptor messenger RNA reveals that the locus coeruleus expresses high levels of tachykinin NK_1-receptor messenger RNA (Maeno et al., 1993). Furthermore, electron microscopy shows substance P-containing fibers form

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axodendritic synapses with catecholamine-containing neurons in the locus coeruleus (Pickel et al., 1979). In addition, substance P increases the firing rate of locus coeruleus neurons (Guyenet and Aghajanian, 1977). Despite the anatomical and pharmacological evidence implicating substance P as a neurotransmitter in the locus coeruleus, there has been no evidence for an action of endogenous substance P in the locus coeruleus during a physiological stimulation.

Given the existence of a substance P innervation of the locus coeruleus, and the possible role of tachykinins in stress, this study examined the role of endogenous substance P in stress-induced expression of c-*fos* in the locus coeruleus. C-*fos* is a protooncogene product that has been extensively used as an index of neuronal activation, since its cellular expression increases in response to many stimuli known to increase the firing rate or metabolic activity of cells (Morgan et al., 1987; Sagar et al., 1988). In the locus coeruleus, induction of c-*fos* expression has been demonstrated in response to a wide variety of noxious stimuli and stressors, including reserpine administration, footshock, water avoidance, restraint, swim stress, hypertonic saline, and conditioned fear (Beck and Fibiger, 1995; Bonaz and Tache, 1994; Ceccatelli et al., 1989; Cullinan et al., 1995; Fritschy et al., 1991; Grant et al., 1992). The effects of tachykinins in the brain have been difficult to study due to a lack of suitable antagonists available. Until recently, tachykinin NK receptor antagonists were not of high affinity nor selectivity for the different tachykinin NK receptors. In addition, the available antagonists were peptides, which would not cross the blood–brain barrier when administered systemically. Recent development of selective, non-peptide tachykinin NK receptor antagonists has made the actions of tachykinins in the brain more amenable to study. In the present study, a series of selective, non-peptide receptor antagonists were tested for their effectiveness in blocking c-*fos* expression in the rat locus coeruleus in response to a restraint stress.

2. Materials and methods

2.1. Animal handling and treatment

Rats (200–280 g) were housed in groups on a 12 h light/12 h dark cycle with food and water available ad libitum. Twenty min following an i.p. injection of a tachykinin NK_1 receptor antagonist or vehicle, some rats were restrained in a ventilated plexiglass tube in which they could not turn around. Rats were released from restraint after 1 h and sacrificed 2 h later. All rats were sacrificed 3 h and 20 min following injection. Rats were anesthetized with 150 mg/kg pentobarbital given i.p. and perfused transcardially with 100 ml of 0.1 M phosphate-buffered saline (pH 7.4) followed by 500 ml of 0.1 M phosphate buffer containing 4% paraformaldehyde. Brains were removed immediately after perfusion, stored in the same fixative overnight, transferred to increasing amounts of sucrose, and stored in 30% sucrose until sectioned.

2.2. Drugs

Three selective, non-peptide tachykinin NK_1 receptor antagonists and their sources were SR 140333 ((*S*)1-(2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane, chloride) (from Dr. X. Emonds-Alt of Sanofi Recherché), RP 67580 ((3a*R*,7a*R*)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl] perhydroisoindol-4-one) (from Dr. C. Garret of Rhone-Poulenc-Rorer), and LY 306740 ((*R*)-1-[*N*-(2-methoxybenzyl)acetylamino]-3-(1 *H*-indol-3-yl)-2-[*N*-(2-(4-cyclohexylpiperazin-1-yl)-acetyl)amino]propane) (from Dr. S. Iyengar of Lilly Research Laboratories). SR 140333 (M.W. 674.1) was dissolved in 0.01% Tween 20 at a concentration of 1.0 mg/ml. RP 67580 (M.W. 438.57) was dissolved in 0.1 N HCl and subsequently diluted to 2.5 mg/ml with water. LY 306740 (M.W. 632.38 as hydrochloride) was dissolved in water at a concentration of 30 mg/ml of the free salt. Appropriate vehicle injections were used in each experiment.

2.3. Immunohistochemistry

Forty-micrometer thick sections were cut at the level of the locus coeruleus on a sliding microtome and stored in phosphate-buffered saline (PBS). Immunocytochemistry was performed on free-floating sections. Sections were treated with 0.3% hydrogen peroxide for 30 min followed by three washes for 10 min each in PBS. Sections were then incubated in 3% goat serum/0.25% Triton X-100 for 2 h to block nonspecific binding. Sections were then incubated for 48 h at 4°C in c-*fos* primary antibody (Oncogene, Cambridge, MA) diluted in blocking solution at 1:100,000, the optimal concentration as determined in pilot experiments. Following three washes in PBS, a Vectastain Elite ABC horseradish peroxidase kit (Vector, Burlingame, CA) was used for the secondary antibody and avidin–biotin complex steps. The colorimetric detection reaction included ammonium chloride, β-D-glucose, and glucose oxidase, to generate the hydrogen peroxide, and 3,3′-diaminobenzidine tetrahydrochloride as chromagen. The reaction was stopped by 100 mM sodium azide. Sections were mounted on slides, air-dried, dehydrated through graded ethanols, treated with xylenes, and coverslipped with Permount.

All cell counts were performed using bright-field microscopy. The left and right locus coeruleus from two sections at two different levels through the locus coeruleus, for a total of four sections, were analyzed from each rat. C-*fos*-positive cells were counted in each of the four sections and averaged. In a few cases, three sections were counted and averaged if one section was damaged through

the locus coeruleus region. Cells containing a nuclear staining of the brown-black reaction product were considered positive for c-*fos*-like immunoreactivity, and are referred to hereafter as c-*fos* positive or c-*fos* expressing cells. C-*fos* positive cells were counted manually under 400× magnification by an observer blinded as to treatment group. Some slides were also analyzed using a computer-assisted system. In this case, microscope images under 256× magnification were collected with a CCD camera and the images analyzed using the MCID image analysis system. Manual cell counts correlated highly ($r^2 = 0.98$) with computer-assisted counting, but demonstrated better discrimination of cells in close contact, or with very low c-*fos* expression (data not shown). Therefore, in all of the experiments presented, manual cell-counting was employed.

2.4. Statistical analysis

Results are expressed as the mean ± the S.E.M. of the number of c-*fos* positive cells. Statistical analysis was performed on each drug experiment. Student's *t*-test was used for the experiments with SR 140333 and LY 306740. One-way analysis of variance followed by Newman–Keuls post-hoc test was employed in the RP 67580 experiment. Values of $P < 0.05$ were considered significant in all experiments.

3. Results

C-*fos*-positive cells were identified on the basis of the presence of a brown-black reaction product that was confined to the nucleus of locus coeruleus cells. The locus coeruleus was easily identifiable as a group of cells located on the floor of, and just lateral to, the fourth ventricle. In some sections immunohistochemistry was performed for both c-*fos* and tyrosine hydroxylase to verify the location of the locus coeruleus cells (data not shown). The c-*fos* positive nuclei of locus coeruleus cells appeared round, of consistent size and were counted only if confined to the defined region (see Fig. 1). Some other cells in the dorsal pons exhibited c-*fos* immunoreactivity in response to some of the treatments, but were easily differentiated from the locus coeruleus cells on the basis of their appearance and location and were not counted.

Animals that received neither injections nor restraint stress exhibited no c-*fos* positive cells in the locus coeruleus. With the exception of one animal, injection of vehicle or tachykinin NK_1 receptor antagonist alone resulted in the appearance of very few c-*fos* positive cells in the locus coeruleus (15 ± 4, $n = 7$; see Fig. 1A). Therefore, only a limited number of injection alone animals were included in subsequent experiments. In contrast, restraint stress resulted in a significant expression of c-*fos* in the locus coeruleus (Fig. 1B).

In the first stress experiment, RP 67580, a selective tachykinin NK_1 receptor antagonist that is more effective in rat than other species (Garret et al., 1991), was administered prior to a restraint stress. It has been previously shown that a dose of 1.5 mg/kg (i.v.), blocks formalin-evoked c-*fos* expression in spinal cord neurons (Chapman et al., 1996). In the present study, administration of 10 mg/kg RP 67580 prior to restraint resulted in an unexpected and significant elevation in the number of c-*fos* expressing cells compared to restraint alone (compare Fig.

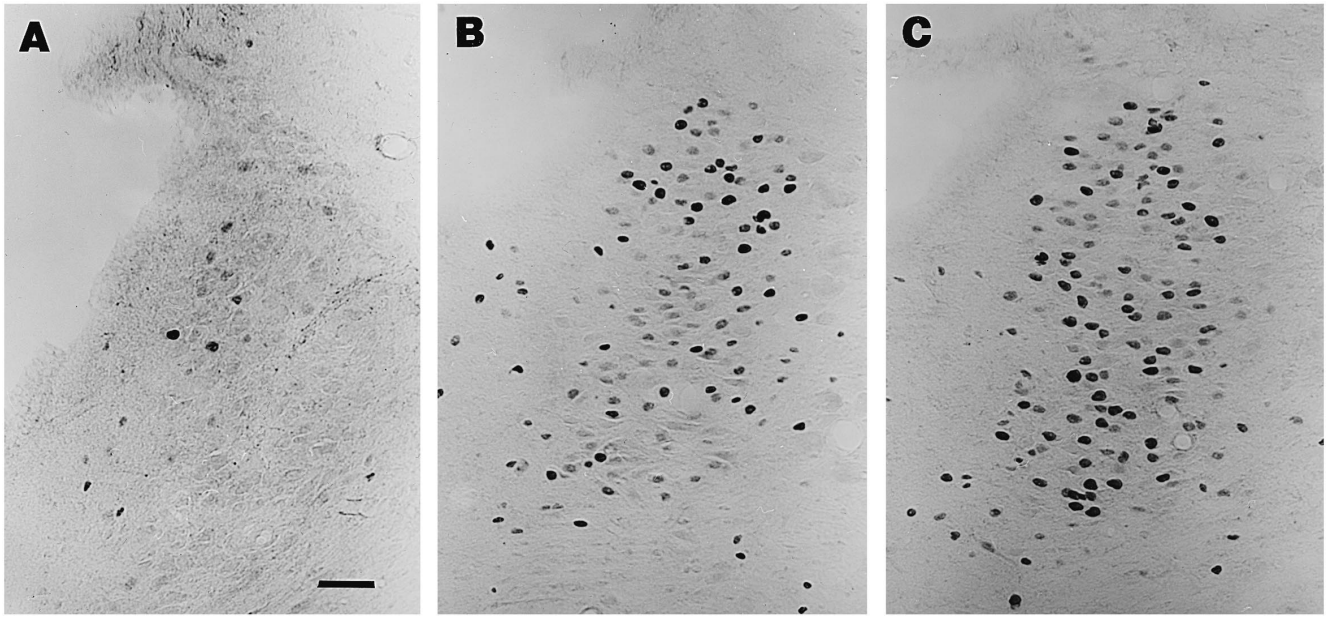

Fig. 1. Representative high-power photomicrographs of c-*fos* immunoreactivity in locus coeruleus following restraint and/or tachykinin NK_1 receptor antagonist. RP 67580 with no restraint (A), 1 h restraint (B), and RP67580 20 min prior to 1 h restraint (C). Scale bar = 50 μm.

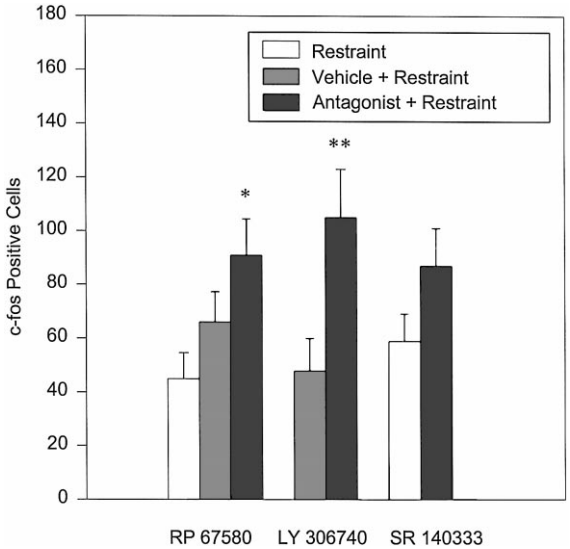

Fig. 2. Summary of the effects of tachykinin NK_1 receptor antagonists on number of restraint-induced c-*fos* expressing cells in the locus coeruleus. Results are expressed as mean ± S.E.M. of number of c-*fos*-positive cells; $n = 4–6$, except for vehicle plus restraint in RP 67580 experiment, where $n = 3$. Significantly different from restraint, $* P < 0.05$, or significantly different from vehicle plus restraint, $** P < 0.05$.

1B and C; Fig. 2). It is possible that an injection prior to restraint would in itself be a stressor and produce an additive effect with restraint on c-*fos* expression. Another group of animals in this experiment was given a vehicle injection prior to restraint. This group demonstrated c-*fos* expression that was not significantly different from restraint alone (Fig. 2). Therefore, the injection procedure per se did not contribute significantly to the number of c-*fos* expressing cells induced by restraint plus drug injection.

In an effort to clarify these results, the chemically distinct tachykinin NK_1 receptor antagonist LY 306740 was administered prior to restraint in the next experiment. This compound has a somewhat higher affinity for human and guinea pig receptors than for rat, but is centrally active when given i.p. in rat models (Hipskind et al., 1996; McCarson and Krause, 1996). Formalin-evoked hindlimb flinches are blocked by i.p. administration of 30 mg/kg LY 306740 (McCarson and Krause, 1996). Therefore, the present studies employed 30 mg/kg LY 306740. Rats receiving a vehicle injection plus restraint again exhibited many c-*fos* positive cells in the locus coeruleus (Fig. 2). Similar to RP 67580, an injection of 30 mg/kg LY 306740 prior to restraint significantly enhanced the number of c-*fos* expressing cells compared to restraint plus vehicle (Fig. 2).

In the final experiment, the selective tachykinin NK_1 receptor antagonist SR 140333 was administered prior to restraint. This compound is efficacious in several species, including rat (Emonds-Alt et al., 1993). SR 140333, when given i.p. at doses of 0.1–3.0 mg/kg, blocks scratching and turning induced by centrally administered tachykinin NK_1 receptor agonists in mice (Jung et al., 1994). In the present experiment, restraint stress again resulted in a large number of c-*fos* expressing cells in the locus coeruleus (Fig. 2). However, 1.0 mg/kg of SR 140333 failed to affect the number of c-*fos* expressing cells compared to restraint alone (Fig. 2), in contrast to the other tachykinin NK_1 receptor antagonists.

4. Discussion

The present study detected c-*fos* expression in locus coeruleus cells 2 h after the completion of restraint, which is consistent with the results of other studies. C-*fos* messenger RNA levels reportedly reach peak values by 1–2 h after onset of restraint (Cullinan et al., 1995; Watanabe et al., 1994) and increases in c-*fos* protein are apparent 1 or 2 h after completion of a 1 h restraint (Ceccatelli et al., 1989; Chen and Herbert, 1995; Palkovits et al., 1995). Untreated animals, only handled at the time they were taken from the cage and given an injection of anesthetic immediately prior to perfusion, demonstrated no c-*fos* in locus coeruleus.

Injections can, under some conditions, induce c-*fos* expression in neurons in the brain (Ceccatelli et al., 1989). In the present experiments, there was very little locus coeruleus c-*fos* expression in animals injected with vehicle or with drug alone. This suggests that injection, and the handling of rats associated with an i.p. injection, was not a stressor of sufficient magnitude to induce locus coeruleus c-*fos* at the time point examined. It is also possible that an injection combined with restraint could produce a greater effect than either produced alone. Therefore, in one experiment, rats were either pretreated with a vehicle injection prior to restraint or restrained without a prior injection. The effect of vehicle injection prior to restraint was not significantly different from restraint alone (Fig. 2). These results provide evidence that the effect of the tachykinin NK_1 receptor antagonist injection prior to stress to enhance locus coeruleus c-*fos* was not due to a combinatorial effect of an injection plus restraint, but rather, to tachykinin NK_1 receptor antagonism.

The tachykinin NK_1 receptor antagonists RP 67580 and LY 306740 both caused an increase in the number of locus coeruleus c-*fos* expressing cells when administered prior to restraint. Given both the presence of tachykinin NK_1 receptors and an excitatory effect of substance P in the locus coeruleus, it was predicted that tachykinin NK_1 receptor antagonists would block restraint-induced locus coeruleus activation. In the present study, tachykinin NK_1 receptor antagonists did not block, but rather, enhanced locus coeruleus c-*fos* expression. It is likely that these drugs act at tachykinin NK_1 receptors in other areas of the brain or

act in the periphery when administered systemically as in the present experiments. The overall tachykinin NK_1 receptor antagonist effect on the locus coeruleus could thus be due to an effect on another pathway, overriding the direct effect of locus coeruleus tachykinin NK_1 receptor antagonism under stress conditions. For example, RP 67580 has been shown to block both substance P-induced increases in blood pressure and heart rate (Culman and Unger, 1995). Blocking central tachykinin NK_1 receptor-mediated responses could result in a further activation of the locus coeruleus, as a decrease in blood pressure elicits an increase in locus coeruleus firing rate (Page and Valentino, 1994; Svensson, 1987).

There also exists the possibility that these antagonists are acting at sites other than in the brain. Tachykinin NK_1 receptors and substance P are abundant in the spinal cord and peripheral nervous system (Patacchini and Maggi, 1995). It is important to note that SR 140333 was the only tachykinin NK_1 receptor antagonist that did not enhance restraint-induced locus coeruleus c-*fos*. SR 140333 contains a quaternary amine, which would likely limit its penetration of the blood–brain barrier. This suggests that enhancement of stress-induced locus coeruleus c-*fos* expression is due to a central effect of the tachykinin NK_1 receptor antagonists. However, SR 140333 given i.p., in doses similar to the present study, reportedly blocks the turning behavior caused by intrastriatal substance P administration (Jung et al., 1994). Therefore, the site of action of the tachykinin NK_1 receptor antagonists used in the present study remains an open question.

The role of locus coeruleus neuronal activity in anxiety is controversial. It has been a long-held belief that an increase in locus coeruleus activity augments fearful or anxious responses (Redmond, 1987). There is also evidence to challenge this notion, that suggests the increase in locus coeruleus activity that accompanies anxious or stressful situations is a compensatory, coping mechanism (Weiss et al., 1994). Some studies have examined the effects of tachykinin NK receptor antagonists in animal models of anxiety. RP 67580 shows anxiogenic effects in mice tested in the black-white box behavioral paradigm (Zernig et al., 1993), while centrally administered FK 888 (another tachykinin NK_1 receptor antagonist) demonstrates an anxiolytic effect in mice in the elevated plus maze paradigm (Teixeira et al., 1996). In the present study, centrally acting tachykinin NK_1 receptor antagonists increased the locus coeruleus c-*fos* expression observed following stress. That tachykinin NK_1 receptor antagonists both demonstrate activity in animal models of anxiety, and increase locus coeruleus neuronal activity (as measured by c-*fos* expression in the present work) raises some interesting questions. In light of the above controversy regarding the relationship between locus coeruleus activity and the experience of anxiety, it will be interesting to assess the effects of tachykinin NK_1 receptor antagonists on anxiety in clinical evaluations.

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