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SSR240600 [(*R*)-2-(1-{2-[4-{2-[3,5-Bis(trifluoromethyl) phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide], a Centrally Active Nonpeptide Antagonist of the Tachykinin Neurokinin 1 Receptor: II. Neurochemical and Behavioral Characterization

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ABSTRACT

SSR240600 [(*R*)-2-(1-{2-[4-{2-[3,5-bis(trifluoromethyl)phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide], a new nonpeptide tachykinin neurokinin 1 (NK_1) receptor antagonist, was evaluated against the neurochemical, electrophysiological, and behavioral effects provoked by direct activation of brain tachykinin NK_1 receptors or by stress in guinea pigs. SSR240600 (0.1–10 mg/kg i.p. or p.o.) antagonized the excitatory effect of i.c.v. infusion of [Sar^9,$Met(O_2)^{11}$]substance P (SP) on the release of acetylcholine in the striatum of anesthetized and awake guinea pigs. This antagonistic action was still observed after repeated administration of SSR240600 (5 days, 10 mg/kg p.o., once a day). SSR240600 (10 mg/kg i.p.) inhibited the phosphorylation of the cAMP response element-binding protein in various brain regions induced by i.c.v. administration of [Sar^9,$Met(O_2)^{11}$]SP. In slice preparations, neuronal firing of the locus coeruleus (LC) neurons elicited by the application of [Sar^9,$Met(O_2)^{11}$]SP was suppressed by SSR240600 at 100 nM. Norepinephrine release in the prefrontal cortex, elicited either by an intra-LC application of [Sar^9,$Met(O_2)^{11}$]SP or by an i.c.v administration of corticotropin-releasing factor, was reduced by SSR240600 (0.3–1 mg/kg and 1–10 mg/kg i.p., respectively). SSR240600 (1–10 mg/kg i.p.) inhibited vocalizations induced in adult guinea pigs by an i.c.v. administration of the NK_1 receptor agonist, GR73632 [D-Ala-[L-Pro^9,Me-Leu^8]substance P(7-11)]. Furthermore, SSR240600 (1–10 mg/kg i.p.) inhibited distress vocalizations produced in guinea pig pups by maternal separation. SSR240600 also reduced maternal separation-induced increase in the number of neurons displaying NK_1 receptor internalization in the amygdala. Finally, SSR240600 counteracted the increase in body temperature induced by isolation stress. In conclusion, SSR240600 is able to antagonize various NK_1 receptor-mediated as well as stress-mediated effects in the guinea pig.

Substance P (SP), via the activation of its preferred target, the tachykinin NK_1 receptor, has been suggested to be involved in the modulation of emotional processes. This idea was initially based on the findings that SP acts as an excitatory neurotransmitter within key neuronal circuits regulating emotional responses, and on the evidence that changes in SP content or release occur in discrete brain regions in response to aversive stimuli (Bannon et al., 1983, 1986; Brodin et al., 1994; Smith et al., 1999; Husum et al., 2001). For example, intermittent foot shocks in rats reduce SP content in the ventral tegmental area (Bannon et al., 1986), olfactory tubercle (Siegel et al., 1984), and several hypothalamic nuclei (Siegel et al.,1987), whereas increased SP concentrations were observed in the medial septum and dentate gyrus (Siegel et al., 1984). Interestingly, chronic treatment with tricyclic antidepressants has been found to decrease SP concentrations in the amygdala, cingulate cortex, nucleus accumbens, or hypothalamus (Shirayama et al., 1996).

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ABBREVIATIONS. SP, substance P; NK_1, neurokinin 1 receptor; SSR240600, (*R*)-2-(1-{2-[4-{2-[3,5-Bis(trifluoromethyl)phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide; ACh, acetylcholine; CREB, cAMP response element binding protein; NE, norepinephrine; LC, locus coeruleus; PFC, prefrontal cortex; CRF, corticotropin-releasing factor; A, anterior; L, lateral; V, ventral; ANOVA, analysis of variance; pCREB, phosphorylated CREB; AUC, area under the curve; GR73632, D-Ala-[L-Pro^9,Me-Leu^8]substance P(7-11); NKP608, (quinoline-4-carboxylic acid [*trans*-(2*R*,4*S*)-1-(3,5-bis-trifluoromethyl-benzoyl)-2-(4-chloro-benzyl)-piperidin-4-yl]-amide); MK-869, 2-(*R*)-(1-(*R*)-3,5-bis(trifluoromethyl)phenylethoxy)-3-(*S*)-(4-fluoro)phenyl-4-(3-oxo-1,2,4-triazol-5-yl)methylmorpholine.

The involvement of SP in the modulation of emotionality has also received considerable support from studies showing that central injection of tachykinin NK_1 receptor agonists produces a range of behavioral and physiological reactions indicative of increased anxiety. Moreover, experiments with selective nonpeptide NK_1 receptor antagonists have indicated that these compounds are effective in animal models of anxiety and depression (for review, see Griebel, 1999). For example, NKP608 has been shown to produce anxiolytic-like activity in the social interaction and social exploration tests in rats (File, 2000; Vassout et al., 2000) and in the stress-induced hyperthermia procedure in mice (Spooren et al., 2002). Moreover, repeated treatment with NKP608 for 5 weeks yielded antidepressant-like activity in the chronic mild stress model in rats (Papp et al., 2000), an effect that is in line with the antidepressant efficacy of another NK_1 receptor antagonist, MK-869, in a double-blind study performed in patients with major depression (Kramer et al., 1998; Rupniak and Kramer, 1999).

In the present paper, we report on the in vivo neuropharmacological profile of a new selective nonpeptide tachykinin NK_1 receptor antagonist, SSR240600 [(*R*)-2-(1-{2-[4-{2-[3,5-bis(trifluoromethyl)phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide] in the guinea pig. The primary objective of this paper was to evaluate the activity of SSR240600 on the neurochemical, electrophysiological, and behavioral effects provoked by direct activation of the brain tachykinin NK_1 receptors and NK_1 receptor agonist-induced 1) release of striatal ACh release, 2) phosphorylation of CREB in various brain regions, 3) firing of NE neurons in LC slice preparations, (4) release of NE in the PFC, and 5) audible vocalization. The second objective was to test the ability of SSR240600 to block stress-induced changes in behavioral and neurochemical responses, using maternal separation-induced distress vocalizations and NK_1 receptor internalization in the amygdala, isolation-induced hyperthermia, and CRF-induced release in cortical NE.

Materials and Methods

Animals

Male Hartley guinea pigs (Harlan, Horst, The Netherlands) weighing from 150 to 521 g were used in microdialysis, immunohistochemistry, electrophysiology, and stress-induced hyperthermia experiments. Female guinea pigs with four 5-day-old pups were used in the maternal separation procedures. Each mother was housed individually with her litter, provided with sawdust. All animals were maintained under standard laboratory conditions (21 ± 1°C) with food (Union pour une alimentation rationelle, Epinay, France) and tap water freely available, and kept on a 12-h light/dark cycle with light onset at 7:00 AM. All procedures have been approved by the Comité d'Expérimentation Animale (Animal Care and Use Committee) of Sanofi-Synthélabo Recherche and were carried out in accordance with French legislation (decree n°. 87-848, October 19, 1987 and an order from April 19, 1988) which implemented the European directive 86/609/EEC.

Effects of SSR240600 in Models of NK_1 Receptor Activation

[Sar^9,$Met(O_2)^{11}$]SP-Induced Release of Striatal Acetylcholine and Cortical Norepinephrine. Guinea pigs were anesthetized with urethane (1.4 g/kg i.p.) and then placed in a stereotaxic frame. Their body temperature was monitored by a rectal probe and adjusted (37 ± 1.1°C) by a homeothermic blanket. The skull and the dura were opened at the level of the striatum to assess ACh release, the medial PFC, and the LC to assess NE release and to perform local application of NK_1 receptor agonist.

The microdialysis probe (length 3 mm for all these regions, except 2 mm for the LC, o.d. 0.5 mm; CMA 12, Carnegie Medicine AB, Stockholm, Sweden) was implanted at the following coordinates referenced to anterior (A) to the interaural line, lateral (L) to the midline, and ventral (V) to the dural surface (Rapisarda and Bacchelli, 1977), striatum (A, 11.8; L, 3.5; V, 6.2), medial PFC (A, 16.6; L, 0.5; V, 5) and LC (A, 5.6; L, 6.5; V, 9.2).

Two days before dialysis measurements, animals were anesthetized with equithezine (4% chloral hydrate, 6% pentobarbital, 4 ml/kg of body weight). Cannula guides were stereotaxically implanted in striatum (the coordinates were A, 11.8; L, 3.5; V, 3.2). On the day of the experiment, animals were placed in a Plexiglas cage; a microdialysis probe (CMA 12, Carnegie Medicine A B, length 3 mm) was inserted into the striatum. After each microdialysis experiment, cannulae placements were confirmed using trypan blue infusion.

ACh Microdialysis Sampling Experiments. The probes were perfused with a gassed Ringer's solution containing 125 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23 mM $NaHCO_3$, and 1.5 mM KH_2PO_4, pH 7.4, at a rate of 2 μl/min using a microinjection pump (CMA-100; Carnegie Medicine). To reduce ACh degradation in the dialysate, 10 μM neostigmine was added to the Ringer's solution perfused in the striatum probe. Microdialysis sampling started 90 min after the probe was placed in the striatum. Serial samples (60 μl) were collected at 30-min intervals. Each treatment was performed after collection of at least four basal ACh values: [Sar^9,$Met(O_2^{11}$]SP (1 and 10 μM) was perfused in the striatum through the probe for 120 min; SSR240600 (0.03, 0.1, and 0.3 mg/kg) was given i.p. (5 ml/kg body weight) 30 min before peptide application. The samples were immediately frozen and stored in a deep freeze (−80°C) no more than 4 days before assay.

Assay of Extracellular ACh Levels. ACh levels were measured in 30-min dialysate samples (60 μl) by a high-performance liquid chromatography system (Waters Instruments, St Quentin en Yvelines, France), as previously described by Steinberg et al. (1995), except for the electrochemical detection system (ESA Coulochem II, Cergy-Pontoise, France). Briefly, the analytical system for ACh included a trapping precolumn and immobilized enzyme reactor (BAS MF-6151; Phymep, Paris, France). The mobile phase-35 mM phosphate buffer (pH 8.5) supplemented with the antibacterial reagent Kathon (5 ml/l; BAS DF-2150; Phymep) was pumped at a flow rate of 0.8 ml/min and replaced with a fresh preparation every 3 days. The enzyme postcolumn reactor converted ACh to hydrogen peroxide, which was electrochemically detected at a platinum electrode (ESA P/N 55-0183) set at 0.3 V. The chromatographic column and enzyme reactor were kept at 35°C.

Time course effect of [Sar^9,$Met(O_2)^{11}$]SP on ACh levels were analyzed by analysis of variance (ANOVA) with repeated measures. Dunnett analysis was used for individual time comparisons. Drug antagonism of the effects of [SAR^9,$Met(O_2)^{11}$]SP was evaluated by comparing the area under the curve during the 120-min intrastriatal application of the peptide. Statistical analysis was carried out by ANOVA followed by Dunnett's *t* test or Duncan's test.

NE Microdialysis Sampling Experiments. After discarding the first 150-min perfusion period, 40-μl perfusate samples were collected at 20-min intervals into Eppendorf microtubes containing 5 μl of 0.1 N $HClO_4$, 1 mM EDTA, and 4 mM Na-metabisulphite. Four baseline samples were collected before intra-LC application of [Sar^9,$Met(O_2)^{11}$]SP. SSR240600 (0.03–0.3 mg/kg) was administered i.p. (5 ml/kg body weight) 30 or 180 min before [Sar^9,$Met(O_2)^{11}$]SP. The samples were immediately frozen and stored in a deep freeze (−80°C) no more than 4 days before assay.

Assay of Extracellular NE Levels. NE levels were measured in 30-μl dialysate samples by a high-performance liquid chromatography system with coulometric detection as previously described

(Marco et al., 1998), except for the mobile phase containing 7% acetonitryl as organic agent. The analytical system consisted of an ESA Coulochem II electrochemical detector equipped with a model 5014 analytical cell (ESA, Chelmsford, MA).

The time course effect of [Sar^9,$Met(O_2)^{11}$]SP on NE levels was analyzed by an ANOVA with repeated measures. Dunnett analysis was used for individual time comparisons. Drug antagonism of the effects of [Sar^9,$Met(O_2)^{11}$]SP were evaluated by comparing the area under the curve during 80 and 120 min following intra-LC application of the SP agonist. Statistical analysis was carried out by ANOVA followed by Dunnett's *t* test or Duncan's test.

[Sar^9,$Met(O_2)^{11}$]SP-Induced Firing of Norepinephrine Neurons in the Locus Coeruleus. Guinea pigs were anesthetized with ketamine (200 mg/kg i.p.) and decapitated. The brain was removed and slices were prepared as previously described by Henderson et al. (1982). Extracellular recordings on guinea pig LC neurons were made by conventional techniques, and spikes were computed on-line through a CED 1401 interface with suitable software (Spike 2; Cambridge Electronic Design Ltd., Cambridge, Cambridgeshire, UK). When viewed with transmitted light under a binocular microscope, the locus coeruleus appeared as a relatively translucent crescent-shaped area on the ventrolateral border of the fourth ventricle. Noradrenergic neurons were identified by their electrophysiological properties [spontaneous firing with a constant rate (0.05–5 Hz) and long-lasting (2 ms) biphasic action potentials], and in almost all cases by the application of 10 μM norepinephrine, which blocks their firing. The use of these electrophysiological and pharmacological criteria minimized the possibility of confusion with non-noradrenergic neighboring neurons (Jung et al., 1996). [Sar9, Met(O2)11]SP (30 nM) was perfused during 1 min, with an interval of 20 min between successive applications, and its effect was expressed as an increase in firing frequency, calculated by subtracting two periods of 100 s each: the mean frequency occurring during a control period from the mean frequency occurring during the agonist response period. SSR240600 was perfused during 100 min and its effect evaluated after 20, 40, 60, 80, and 100 min on the [Sar9, Met(O2)11]SP-induced increase in firing rate of neurons. Statistical analysis was carried out by an ANOVA with repeated measures followed by Dunnett's *t* test.

[Sar^9,$Met(O_2)^{11}$]SP-Induced Phosphorylation of CREB. Guinea pigs received SSR240600 (10 mg/kg i.p.) 3 h before i.c.v. application of [Sar9,$Met(O_2)^{11}$]SP (2 nmol). They were anesthetized with sodium pentobarbital (80 mg/kg i.p.) 15 min after [Sar9,$Met(O_2)^{11}$]SP injection and perfused transcardially with 4% paraformaldehyde. The immunodetection of CREB and phosphorylated CREB (pCREB) was performed on adjacent brain sections (70 μm) using rabbit polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY) directed against CREB (1:2000) or pCREB (1:800), followed by biotinylated secondary anti-rabbit antibodies (Vector Laboratories, Burlingame, CA) and DAB chromogenic reaction (Vector-Laboratories). The CREB- and pCREB-immunoreactive signals were quantified with an image analysis system (Samba Technologies, France, Meylan, France) by counting the number of CREB- or pCREB-positive cells within the prefrontal cortex (infralimbic), the ventrolateral septum, and the basolateral amygdala.

GR73632-Induced Vocalizations in Adult Guinea Pigs. A guide cannula was inserted into the third cerebral ventricle (A, 6; L, 0; V, 6.2) of anesthetized guinea pigs. Up to 10 days after surgery, animals were injected i.c.v. with GR73632 (0.2 nmol/5 μl/2.5 min), and long-lasting audible vocalizations were recorded for 30 min. Kruskal-Wallis test analysis was subsequently applied to determine which dose was significantly different from vehicle.

Effects of SSR240600 on Stress-Induced Changes of Behavioral and Neurochemical Responses

Maternal Separation-Induced Distress Vocalizations in Guinea Pig Pups. The procedure was adapted from that described by Molewijk et al., (1996). Briefly, from day 9 of age, pups entered two pretest sessions (with 2-day intervals) consisting of 5 min of isolation in a sound-attenuated cage equipped with white noise and white illumination, and the durations of their vocalizations were recorded by the experimenter. Immediately after the 5-min isolation, the subjects were returned to their mothers and littermates. Pups emitting vocalizations during at least 120 s entered subsequent drug experimentation. Each pup was tested with vehicle and two dose levels of a compound with a washout period of 3 days between each treatment. SR240600 (1–10 mg/kg) was administered 30 min before the 5-min experiment. A repeated-measures ANOVA was used with an appropriate covariance structure for vocalization duration analysis. Dunnett's *t* test analysis was subsequently applied to determine which dose was significantly different from vehicle.

NK_1 Receptor Internalization in the Amygdala. Separation and drug treatments were performed as described above. Five minutes after maternal separation, guinea pig pups were anesthetized with pentobarbital (80 mg/kg i.p.) and perfused transcardially with heparinized saline, followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline (pH = 7.4). Brains were removed and allowed to postfix overnight in 4% paraformaldehyde. Fifty-micrometer coronal slices were then serially sectioned from each brain at the level of the amygdala using a Vibratome. Immunohistochemical analysis of NK_1 receptor-positive neurons was performed on free-floating tissue sections as previously described (Steinberg et al., 2001) using a rabbit NK_1 receptor antibody and a (Cy3)-conjugated donkey anti-rabbit IgG (Chemicon International, Temecula, CA). After washing in phosphate-buffered saline, sections were mounted, air-dried, dehydrated, and covered with Polymount. Serial sections were viewed at the lateral and the anterobasolateral amygdala using a Leica TCS 4D confocal scanning system on a Leica DMIRB microscope (Leica, Wetzlar, Germany). For each animal, NK_1 receptor internalization was quantified as previously described (Steinberg et al., 2001). Briefly, for each slice, single optical sections (63× objective and 1.8 numeric zoom) of eight NK_1 receptor-positive neurons were taken and six to eight consecutive sections were analyzed for each animal. The images (512 × 512 pixels) were taken through the center of the cell body so that they included the nucleus. Two independent experimenters, unaware of the treatment, determined the presence or absence of NK_1 receptor endocytosis in each scanned cell. For each neuron, NK_1 receptors were considered as internalized when the immunolabeling was predominantly intracytoplasmic in the form of bright immunofluorescent particles as opposed to neurons with non-internalized NK_1 receptors that exhibited a uniform labeling on the cell surface. All counts were expressed as the percentage of NK_1-immunoreactive neurons that contained internalized receptors. The mean ± S.E.M. percentage of neurons showing NK_1 receptor internalization was calculated for each treatment group, and comparisons were performed using Student's *t* test.

Isolation-Induced Hyperthermia. The basal rectal temperature of handled guinea pigs (two per cage) was measured with a telethermometer (Ellab DM 852; Ellab, Roedowe, Denmark). Animals were then removed from their home cage and placed individually in a small transparent plastic cage (25 × 15 × 27 cm). Temperature was measured again twice at 15-min intervals. Isolation yielded an enhanced body temperature that putatively reflects a stress-induced anxiogenic response. SSR240600 (3 and 10 mg/kg i.p.) was administered 60 min before basal temperature measurement. Data were analyzed by two-way ANOVA (time × treatment) with repeated measures, followed by Dunnett's *t* test.

CRF-Induced Release in Cortical NE. See microdialysis experiments above for details. SSR240600 (3 and 10 mg/kg) and the reference CRF_1 receptor antagonist, antalarmin (30 mg/kg), were administered i.p. (5 ml/kg body weight) 180 min before an i.c.v. pneumatic ejection of CRF. Time course effects of CRF on NE levels were analyzed by an ANOVA with repeated measures. Dunnett's analysis was used for individual time comparisons. Drug antagonism of the effects of CRF were evaluated by comparing the area under the curve during 80 and 120 min following injection of the peptide.

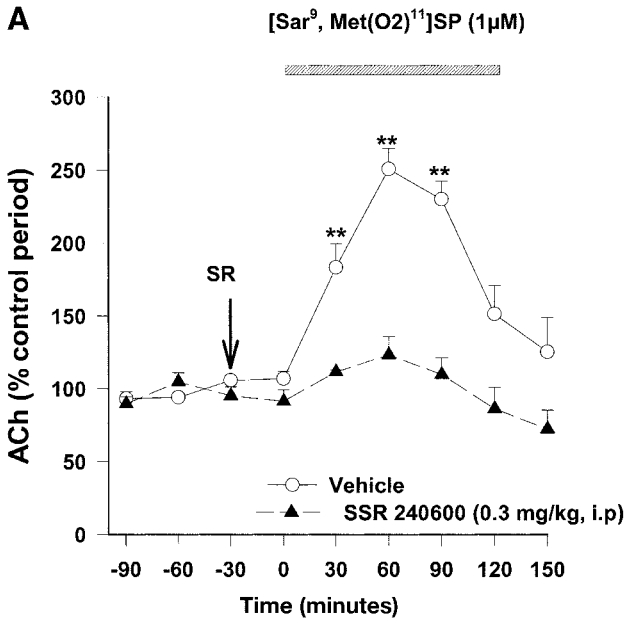

Fig. 1. A, effect of SSR240600 (0.3 mg/kg i.p.) on [Sar^9,Met(O$_2$)^{11}]SP-induced striatal ACh release in anesthetized guinea pigs. The changes in ACh levels are expressed as a percentage of the mean value of the two basal samples before treatment. Each data point represents the mean value ± S.E.M. of four to nine animals. ⋆⋆, $p < 0.01$ compared with control group by ANOVA with repeated measures and Dunnett's t test. B, reversal of [Sar^9,Met(O$_2$)^{11}]SP-induced ACh release by SSR240600 given i.p. 30 min before the agonist. The data are the mean AUC ± S.E.M of four to nine animals for the 120-min application of [Sar^9,Met(O$_2$)^{11}]SP. ⋆, $p < 0.05$, ⋆⋆, $p < 0.01$ compared with the control group by ANOVA followed by Dunnett's t test.

Statistical analysis was carried out by ANOVA followed by Dunnett's t test or Duncan's test.

Drugs

SSR240600 and antalarmin were synthesized by Sanofi-Synthélabo (Montpellier, France). [Sar^9,Met(O$_2$)^{11}]SP and CRF (Novabiochem V W R, Fontenay sous Bois, France) were dissolved in water and Ringer's solution, respectively. SSR240600 and antalarmin were suspended with 0.01% Tween 80 in distilled water and administered i.p. or orally in a volume of 5 or 20 ml/kg body weight for guinea pig adults and pups, respectively.

Results

Effects of SSR240600 in Models Involving Direct Activation of Tachykinin NK$_1$ Receptors

Blockade of [Sar^9,Met(O$_2$)^{11}]SP-Induced Release of Striatal ACh. In anesthetized guinea pigs, the intrastriatal application of [Sar^9,Met(O$_2$^{11}]SP (1 μM) produced a rapid increase in extracellular ACh levels (Fig. 1A), an effect that reached statistical significance between 30 and 90 min after [Sar^9,Met(O$_2$^{11}]SP perfusion (maximal increase at 60 min,

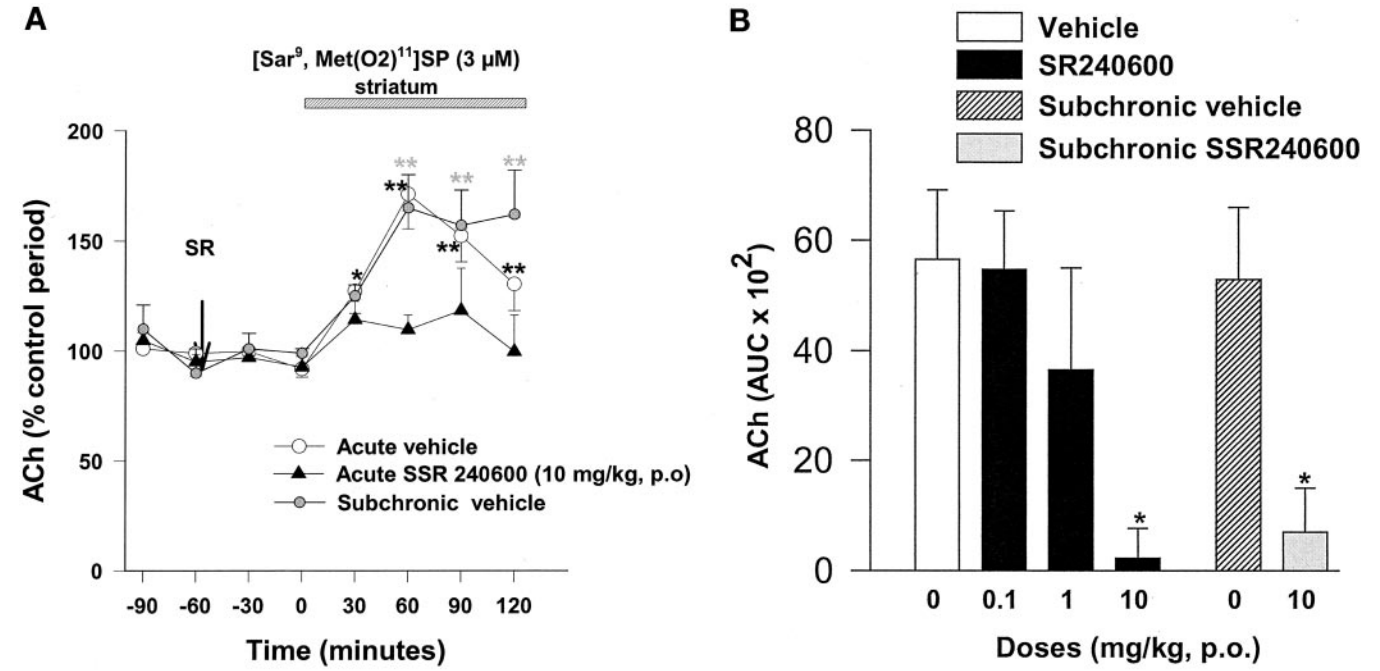

Fig. 2. A, effect of oral administration of SSR240600 (10 mg/kg) on [Sar^9,Met(O$_2$)^{11}]SP-induced striatal ACh release in freely moving guinea pigs. The changes in ACh levels are expressed as a percentage of the mean value of the two basal samples before treatment. Each data point represents the mean value ± S.E.M. of six to seven animals. ⋆⋆, $p < 0.01$ compared with the control group by ANOVA with repeated measures and Dunnett's t test. B, reversal of [Sar^9,Met(O$_2$)^{11}]SP-induced ACh release by SSR240600 given p.o. 60 min before the agonist in acute or subchronic (5 days SSR240600 administration, 10 mg/kg p.o., once a day) groups. The data are the mean AUC ± S.E.M. of four to seven animals for the 120-min application of [Sar^9,Met(O$_2$)^{11}]SP. ⋆, $p < 0.05$ compared with the respective control group by ANOVA followed by Duncan's test.

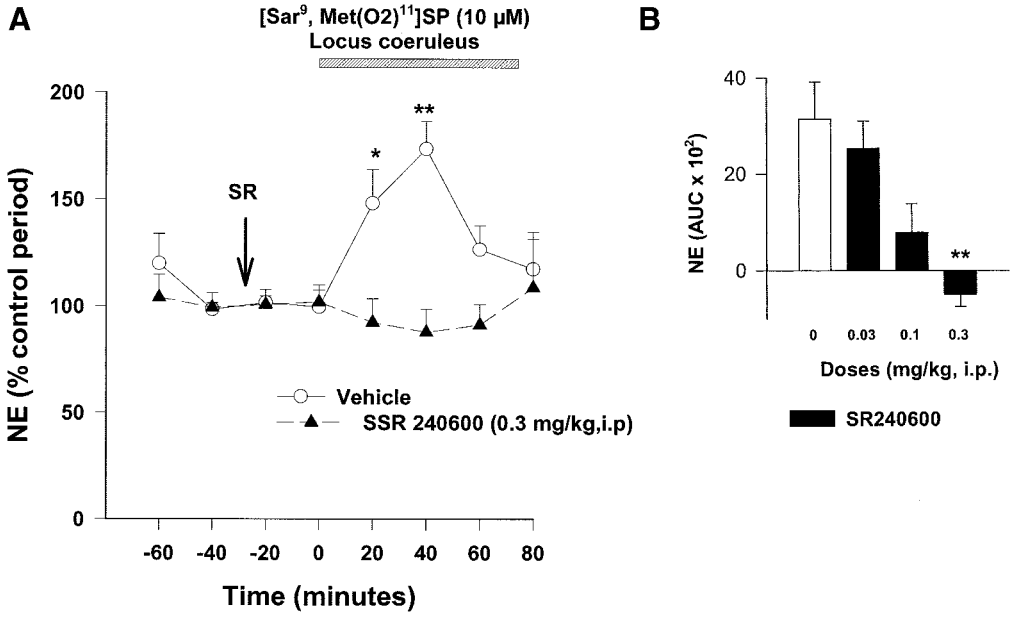

Fig. 3. A, effect of intra-LC application of [Sar^9,Met(O$_2$)^{11}]SP on PFC NE release. The changes in NE levels are expressed as a percentage of the mean value of the four basal samples before treatment. Each data point represents the mean value ± S.E.M of five animals. ⋆, $p < 0.05$, ⋆⋆, $p < 0.01$ compared with the control group by ANOVA with repeated measures and Dunnett's t test. B, reversal of [Sar^9,Met(O$_2$)^{11}]SP-induced NE release by SSR240600 given i.p. 30 min before the agonist. The data are the mean AUC ± S.E.M. of five animals for the 80 min of the NK$_1$ agonist application injection. ⋆⋆, $p < 0.01$ compared with the control group by ANOVA followed by Dunnett's t test.

+151 ± 14%, $p < 0.01$, $n = 9$). SSR240600 (0.3 mg/kg i.p.) injected 30 min before the administration of [Sar^9,Met(O$_2$)^{11}]SP significantly antagonized this effect (Fig. 1A). The effect of SSR240600 was dose-dependent as shown by the area under the curve during the 120 min sampling period of peptide application (Fig. 1B). In freely moving guinea pigs, 3 µM [Sar^9,Met(O$_2$)^{11}]SP produced a rapid increase in extracellular striatal ACh levels (Fig. 2A), an effect that was significant at 60 min after [Sar^9,Met(O$_2^{11}$]SP (maximal increase at 60 min, +71 ± 16%, $p < 0.01$, $n = 7$). SSR240600 (10 mg/kg), injected orally 60 min before the administration of the peptide, antagonized [Sar^9,Met(O$_2$)^{11}]SP-evoked striatal ACh release (Fig. 2A). The dose above 1 mg/kg p.o. was significantly effective, as revealed by the area under the curve during the 120-min sampling period of the peptide application (Fig. 2B). The excitatory effect of [Sar^9,Met(O$_2$)^{11}]SP on striatal ACh release in subchronic vehicle-treated animals (5 days, once a day) was comparable with that of acute vehicle-treated animals (maximal increase at 60 min, +65 ± 15%, $p < 0.01$, $n = 4$) (Fig. 2A). As revealed by the area under the curve (Fig. 2B), the inhibitory effect of SSR240600 was still present in subchronic-treated animals (10 mg/kg orally, 5 days, once a day).

Blockade of [Sar^9,Met(O$_2$)^{11}]SP-Induced Release of Cortical NE. In anesthetized guinea pigs, the local application of 10 µM [Sar^9,Met(O$_2$)^{11}]SP into the LC area increased cortical NE efflux with maximal increase at 40 min (+73 ± 11%, $p < 0.01$, $n = 5$). SSR240600 (0.3 mg/kg i.p.) significantly blocked this effect (Fig. 3A). As revealed by the area under the curve during the 80-min sampling period of [Sar^9,Met(O$_2$)^{11}]SP application (Fig. 3B), the inhibitory effect of SSR240600 (0.03–0.3 mg/kg i.p) was statistically significant ($p < 0.01$) at the dose above 0.1 mg/kg i.p.

Blockade of [Sar^9,Met(O$_2$)^{11}]SP-Induced Activation of NE Neurons in the LC. [Sar9, Met(O2)11]SP applied during 1 min at 30 nM induced an average increase of 2.36 ± 0.49 Hz of LC neuron firing frequency. This effect was time dependently antagonized by perfusion with 100 nM SSR240600, reaching significant values at 80 ($p < 0.05$) and 100 ($p < 0.01$) min (see table 1).

Blockade of [Sar^9,Met(O$_2$)^{11}]SP-Induced CREB Phosphorylation in Guinea Pig Brain. The i.c.v. administration of [Sar^9,Met(O$_2$)^{11}]SP markedly and significantly ($p < 0.05$ versus vehicle-treated animals, $n = 4$) increased the number of pCREB-positive cells in the PFC (infralimbic part, 204%), the ventrolateral septum (350%), and the basolateral amygdala (243%). This stimulatory effect was totally blocked ($p < 0.05$ versus [Sar^9,Met(O$_2$)^{11}]SP) by pretreatment with SSR240600 (10 mg/kg, $n = 3$) (Fig. 4). In contrast, none of these treatments significantly affected the number of CREB-immunoreactive cells in the brain regions analyzed (Fig. 5).

Blockade of GR73632-Induced Vocalizations in Adult Guinea Pigs. The i.c.v infusion of the NK$_1$ receptor agonist GR73632 (0.2 nmol) elicited long-lasting audible vocalizations in adult guinea pigs which were dose dependently abolished by pretreatment with SSR240600 (1–10 mg/kg, i.p.) given 180 min before the SP agonist (Fig. 5).

TABLE 1
Time-dependent antagonism by SSR240600 (100 nM) of [Sar^9,Met(O$_2$)^{11}]SP-induced increase in firing frequency ($n = 4$)

	SSR240600				
Control	20 min	40 min	60 min	80 min	100 min
2.36 ± 0.49 Hz	2.15 ± 0.53 Hz	2.15 ± 0.96 Hz	1.16 ± 0.45 Hz	0.30* ± 0.15 Hz	0.18** ± 0.15 Hz

* $p < 0.05$, ** $p < 0.01$ (ANOVA with repeated measures followed by Dunnett's test).

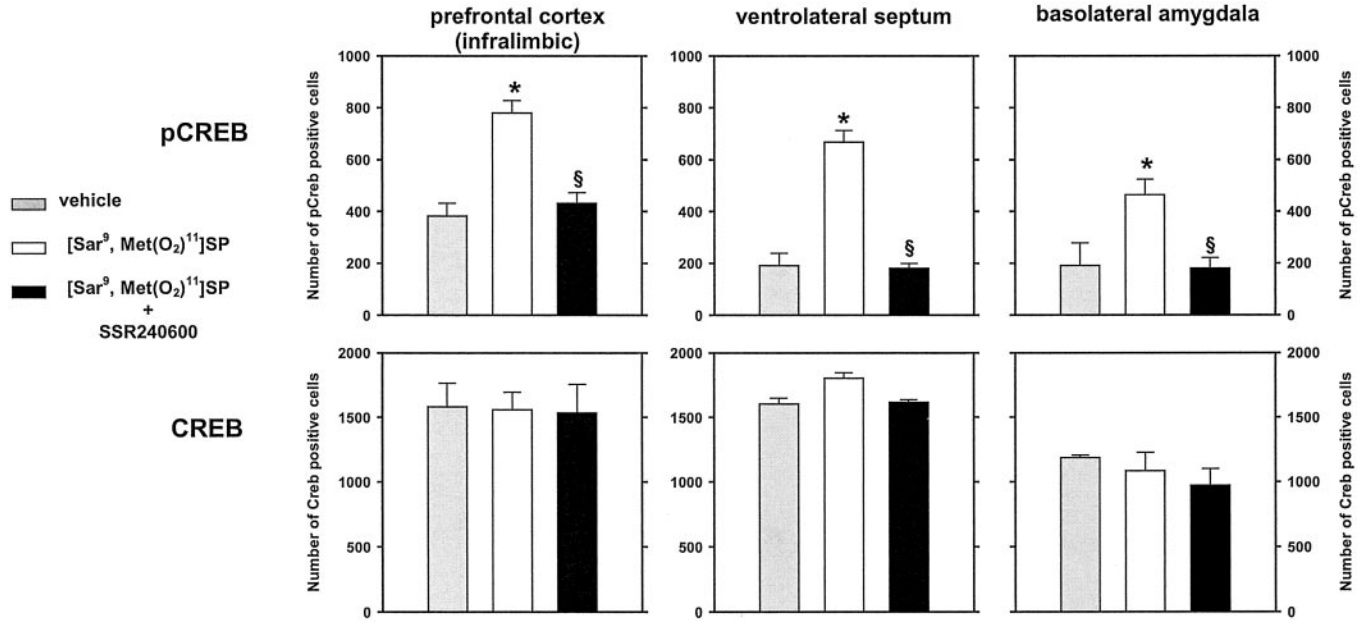

Fig. 4. Effect of SSR240600 on [Sar^9,Met(O$_2$)^{11}]SP-induced CREB phosphorylation in guinea pig brain. Guinea pigs were injected (i.c.v.) with vehicle ($n = 3$) or [Sar^9,Met(O$_2$)^{11}]SP (2 nmol, $n = 4$), or were treated with SSR240600 (10 mg/kg i.p., $n = 3$) 180 min before [Sar^9,Met(O$_2$)^{11}]SP administration. Data are mean ± S.E.M. of the number of pCREB- or CREB-immunoreactive cells counted in the indicated brain regions. ⋆, $p < 0.05$ versus vehicle-treated animals; §, $p < 0.05$ versus [Sar^9,Met(O$_2$)^{11}]SP-treated animals.

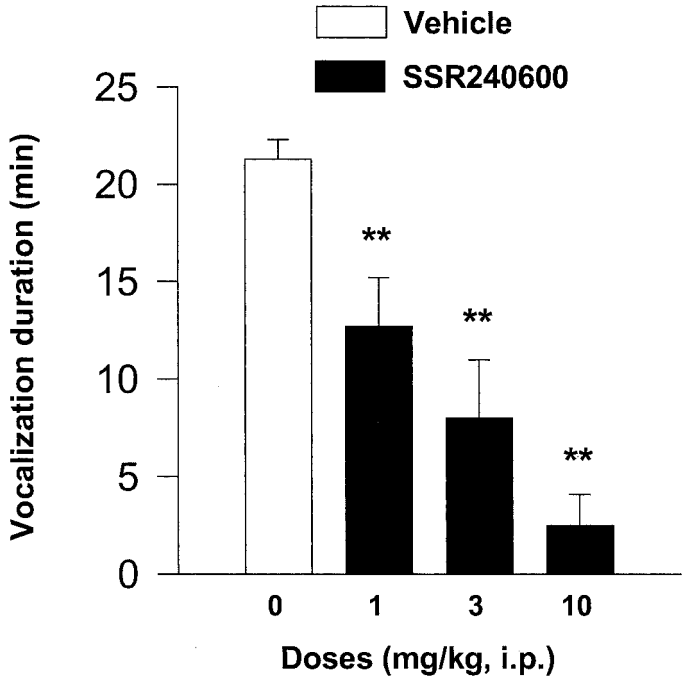

Fig. 5. Effects of SSR240600 on the duration of vocalization induced by infusion of the SP agonist GR73632 (0.2 nmol, i.c.v.) in guinea pigs. SSR240600 was administered intraperitoneally 180 min before the infusion. Data are the mean value ± S.E.M. of 8 to 17 animals. ⋆⋆, $p < 0.01$ compared with the control group by the Kruskal-Wallis test.

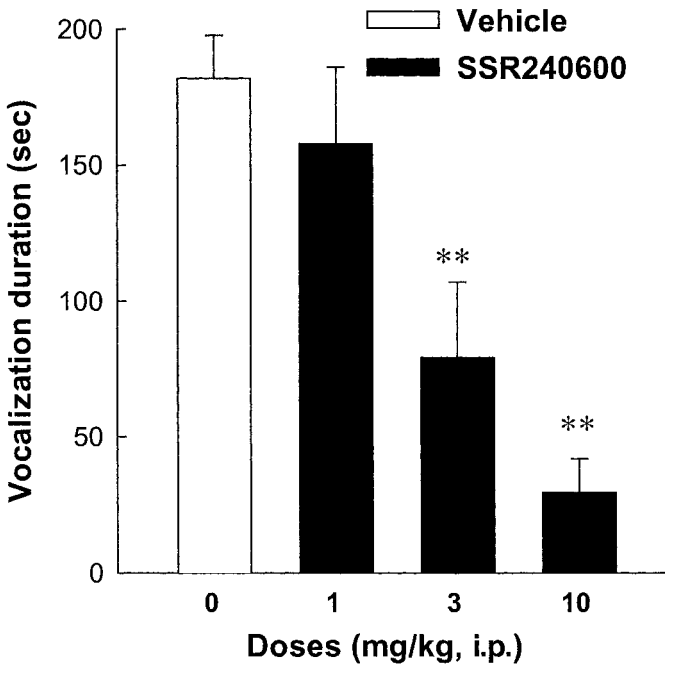

Fig. 6. Effects of SSR240600 on the duration of isolation-induced calls of guinea pig pups. Data are expressed as mean ± S.E.M. of 7 to 55 animals per group. ⋆⋆, $p < 0.01$ compared with the vehicle control group by ANOVA with repeated measures followed by Dunnett's t test.

Effects of SSR240600 on Stress-Induced Changes of Behavioral and Neurochemical Responses

Maternal Separation-Induced Distress Vocalizations in Guinea Pig Pups. In guinea pig pups, the administration of SSR240600 (3 and 10 mg/kg i.p.) 30 min before maternal separation dose dependently and completely inhibited distress vocalizations ($p < 0.01$) (Fig. 6).

NK$_1$ Receptor Internalization in the Amygdala. As we already reported, maternal separation of guinea pig pups was found to produce endocytosis of NK$_1$ receptors in the amygdala, as assessed by immunocytochemical techniques (Steinberg et al., 2001). NK$_1$ receptor immunoreactivity in cell bodies of the amygdala was mostly concentrated on the neuronal membrane in nonseparated guinea pig pups, whereas in separated animals, a majority of neurons exhibited bright immunofluorescent particles within the cyto-

plasm indicating NK_1 receptor endocytosis (not shown). Semiquantitative analysis indicates that in separated animals treated with vehicle, 72.4 ± 2.5% of neurons in the amygdala (mean ± S.E.M. of 192 neurons from four animals) have internalized NK_1 receptors, whereas in SSR240600-treated animals, the extent of NK_1 receptor internalization decreased to 45.1 ± 8.7% (mean ± S.E.M. of 224 neurons from four animals, $p < 0.05$) and 26.7 ± 3.7% (mean ± S.E.M. of 221 neurons from four animals, $p < 0.01$) at 3 and 10 mg/kg i.p., respectively.

Isolation-Induced Hyperthermia. SSR240600 reduced the rise in body temperature following isolation stress (Fig. 7). This effect reached statistical significance at both time periods at 10 mg/kg i.p. ($p < 0.01$), and at 15 min at 3 mg/kg ($p < 0.01$).

Blockade of CRF-Induced Release in Cortical NE. The i.c.v. injection of CRF (3 μg/2 μl) increased cortical NE efflux with a maximal increase at 120 min (+48 ± 9%, $p < 0.01$, $n = 18$). SSR240600 (10 mg/kg i.p., 180 min pretreatment) and antalarmin (30 mg/kg i.p.) significantly blocked the excitatory effect of CRF on cortical NE release (Fig. 8).

Discussion

Our studies have demonstrated that SSR240600 (Emonds-Alt et al., 2002) is a potent orally active nonpeptide antagonist of the tachykinin NK_1 receptor in the brain. SSR240600 is able to block various neurochemical, electrophysiological, and behavioral effects provoked by direct activation of brain tachykinin NK_1 receptors by specific agonists or following stress. Behavioral experiments also show that, like other NK_1 receptor antagonists, SSR240600 displays a profile that is consistent with anxiolytic- and antidepressant-like actions.

As demonstrated previously in rats (Steinberg et al., 1995), local application of the NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]SP produced a marked increase of striatal ACh release in guinea pigs, as assessed by microdialysis. This is consistent with the presence of NK_1 receptors in the striatum of this species (Saffroy et al., 1994). SSR240600, administered acutely either by the intraperitoneal or oral route, attenuated [Sar^9,$Met(O_2)^{11}$]SP-induced striatal ACh release in anesthetized or freely moving animals. This effect was still apparent following daily administration of SSR240600 for 5 days (10 mg/kg p.o., once a day). Collectively, these data demonstrate that SSR240600 is an orally active, brain-penetrant NK_1 receptor antagonist that does not show tolerance to its activity after repeated administration.

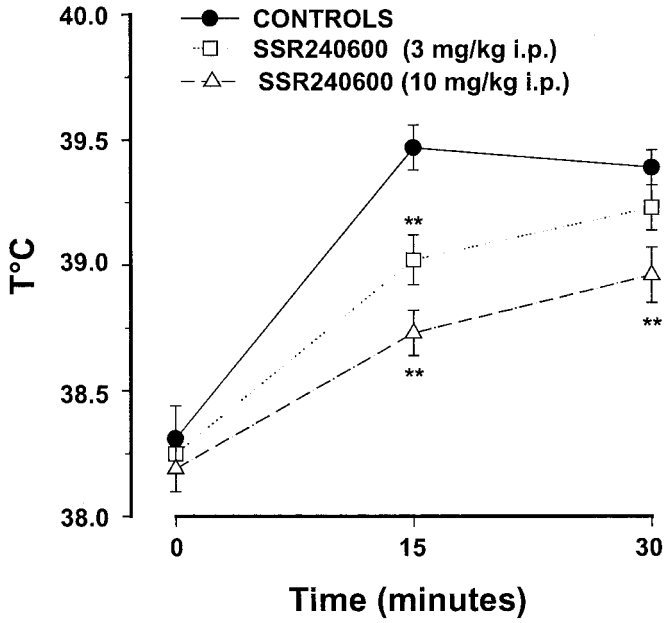

Fig. 7. Effects of SSR240600 on isolation stress-induced hyperthermia in rats. Data represent mean ± S.E.M. **, $p < 0.01$ (Dunnett's t test). $n = 10$.

Since SP was proposed to act as a neuromodulator of neuronal circuits involved in stress (Bannon et al., 1983, 1986; Hahn and Bannon, 1999), we sought to determine whether SSR240600 would counteract NK_1 receptor agonist-induced activation of LC PFC NE systems. When applied to LC guinea pig slices, SSR240600 inhibited [Sar^9,$Met(O_2)^{11}$]SP-induced increase in firing of NE LC neurons. The onset of this effect was slow since complete blockade of the agonist response required about 80 min of drug application; a similar observation has been made with other nonpeptide NK_1 receptor/receptor antagonists (Jung et al., 1996). The control of the excitatory effect of [Sar^9,$Met(O_2)^{11}$]SP on the NE system was also found in vivo since SSR24600 totally blocked the NE release in the PFC induced by intra-LC application of the agonist. These results are consistent with the location of NK_1 receptors on NE neurons of the LC and the presence of SP-containing fibers forming axodendritic synapses in the LC region of rodents (Pickel et al., 1979; Hahn and Bannon, 1999). Moreover, our data confirm the excitatory effect of NK_1 receptors on ascending LC noradrenergic pathways in guinea pigs (Saffroy et al., 1994; Bert et al., 2002) and rats (Hahn and Bannon, 1999; Chen et al., 2000), and corroborate the role of endogenous SP in stress-induced activation of LC neurons (Hahn and Bannon, 1999; Maubach et al., 2002).

The present study also provides the first direct evidence of an interplay between the SP/NK_1 receptor and the CRF/CRF1 receptor systems to enhance the release of NE in the PFC. Cortical NE release induced by intracerebral injection of CRF was blocked by prior administration of SSR240600 to a similar extent as with the CRF1 receptor antagonist, antalarmin. The idea that SSR240600 blocked CRF effect by competitive antagonism can be ruled out since SSR240600 has no affinity for CRF receptors (Emonds-Alt et al., 2002). This raises the possibility that CRF provokes excitatory effect on the PFC-LC system by stimulating endogenous SP release. Several studies have demonstrated that CRF administered i.c.v. acts directly on the LC (Valentino et al., 1983, 1991), probably via CRF-containing terminals that make contact with many presynaptic elements in the LC. Moreover, it is possible that CRF releases other neurotransmitters, including neuropeptides, by acting presynaptically to exert excitatory effects on LC neurons on a postsynaptic level (Van Bockstaele et al., 1996; Steinberg et al., 2001). It is important to note that the blockade of NK_1 receptors by SSR240600 did not affect basal levels of ACh and NE in the striatum or in the prefrontal cortex, suggesting a lack of tonically active SP control on ACh and NE release in anesthetized or awake animals.

Immunohistochemical data, using pCREB as a marker of neuronal activity (Deisseroth et al., 1996; Finkbeiner et al.,

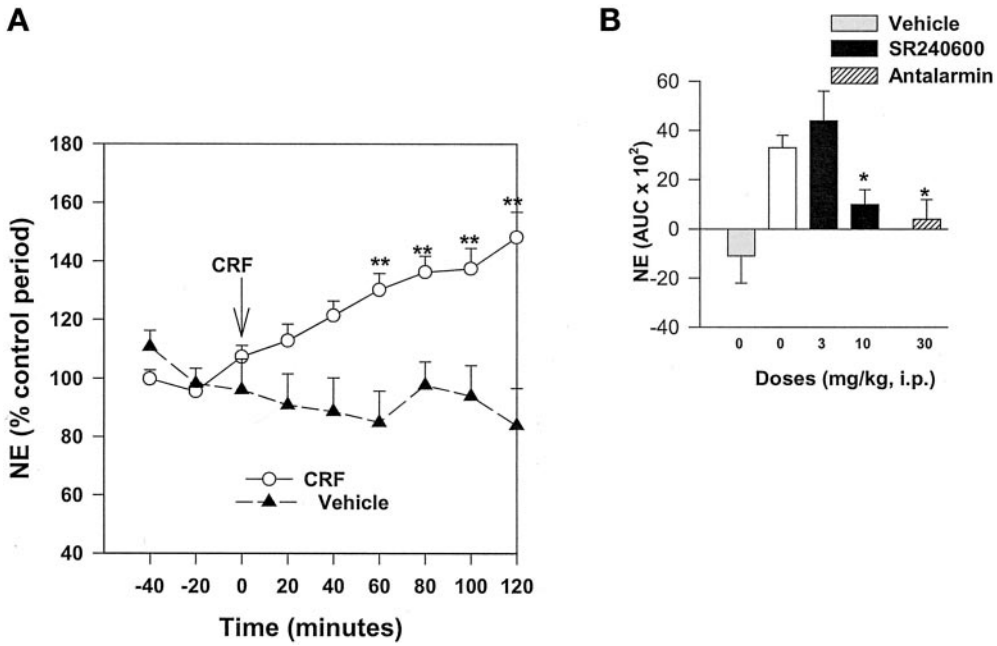

Fig. 8. A, effect of i.c.v. injection of CRF (3 μg, 2 μl) on PFC NE release. The changes in NE levels are expressed as a percentage of the mean value of the four basal samples before treatment. Each data point represents the mean value ± S.E.M of five animals. ⋆⋆, $p < 0.01$ compared with the control group by ANOVA with repeated measures and Dunnett's t test. B, reversal of [Sar^9,Met(O$_2$)^{11}]SP-induced NE release by SSR240600 and antalarmin given i.p., 180 or 30 min before the agonist, respectively. The data are the mean AUC ± S.E.M. of five animals for the 80 min of the peptide application. ⋆, $p < 0.05$ compared with the control group by ANOVA followed by Duncan's test.

1997), show that, besides the PFC, other brain regions involved in the modulation of stress responses, such as the amygdala and septum, were stimulated by cerebral administration of [Sar^9,Met(O$_2$)^{11}]SP. Here, [Sar^9,Met(O$_2$)^{11}]SP likely stimulated CREB phosphorylation as indicated by the increase in the number of pCREB-positive cells without affecting CREB labeling. The administration of SSR240600 reduced [Sar^9,Met(O$_2$)^{11}]SP-induced pCREB expression in these regions, indicating that induction of pCREB was mediated by NK$_1$ receptors. The regional pattern of pCREB labeling following i.c.v. [Sar^9,Met (O$_2$)^{11}]SP administration was similar to that described for Fos studies with the same dose of the NK$_1$ receptor agonist. More precisely, mapping studies demonstrate that SP-containing cell bodies and postsynaptic NK$_1$ receptors are highly expressed in brain regions implicated in emotional processes such as the amygdala, hypothalamus, hippocampus, and periaqueductal gray matter (Otsuka and Yoshioka, 1993; Petitet et al., 1993; Saffroy et al., 1994; Yip and Chahl, 1999). The finding that a NK$_1$ receptor agonist increased CREB phosphorylation in these regions strengthens the role played by NK$_1$ receptors in emotional processes.

Previous studies showed that central application of NK$_1$ receptor agonists in guinea pigs or gerbils produces a range of fear reactions, including sonic vocalization or foot-tapping (Kramer et al., 1998; Rupniak and Kramer, 1999; Ballard et al., 2001). These behaviors are generally blocked by pretreatment with NK$_1$ receptor antagonists, but also by the administration of clinically effective antidepressants and anxiolytics (Kramer et al., 1998; Rupniak et al., 2000). Here, we found that the administration of SSR240600 dose dependently abolished vocalizations elicited in adult guinea pigs by central infusion of the NK$_1$ receptor agonist, GR73632. Moreover, SSR240600 completely suppressed distress vocalizations displayed by guinea pig pups after maternal separation. This latter model was reported to be sensitive to antidepressants but has also proven to be useful for the screening of anxiolytic drugs (Molewijk et al., 1996; Kramer et al., 1998; Rupniak et al., 2000; Steinberg et al., 2001). As such, the activity of SSR240600 in this paradigm may indicate that it may be endowed with anxiolytic-like properties. This idea is substantiated by the finding that SSR240600 counteracted the increase in body temperature following isolation stress. The basolateral amygdala, where endogenous SP was shown to be released during different stress conditions, including maternal separation (Kramer et al., 1998; Rupniak and Kramer, 1999), may represent a potential site of action for the effects of SSR240600 on emotional responses. This is evidenced by the findings that 1) maternal separation increased the extent of NK$_1$ receptor internalization in this brain region, a cellular response prevented by prior administration of SSR240600 or another NK$_1$ receptor antagonist (Kramer et al., 1998; Smith et al., 1999), and 2) maternal separation-induced vocalizations were inhibited by an intra-amygdala injection of an NK$_1$ receptor antagonist (Boyce et al., 2001) .

In conclusion, the present study clearly demonstrates that SSR240600 is a potent brain-penetrant antagonist of the tachykinin NK$_1$ receptor able to inhibit various neurochemical, electrophysiological, and behavioral effects provoked by direct activation of tachykinin NK$_1$ receptors by specific agonists. Furthermore, our study confirms that blockade of brain tachykinin NK$_1$ receptors by a specific antagonist is able to reduce neurochemical and behavioral alterations provoked by stress. In addition, this latter effect could be at least partially related to an interplay between the SP/NK$_1$ receptor and the CRF/CRF1 receptor systems.

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