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ELECTROPHYSIOLOGICAL, BEHAVIOURAL AND BIOCHEMICAL EVIDENCE FOR ACTIVATION OF BRAIN NORADRENERGIC SYSTEMS FOLLOWING NEUROKININ NK_3 RECEPTOR STIMULATION

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Abstract—The objective of the present *in vitro* and *in vivo* experiments was to examine the involvement of neurokinin NK_3 receptors in the regulation of the noradrenergic function in gerbils and guinea-pigs. Application of senktide, a peptide NK_3 receptor agonist, on guinea-pig locus coeruleus slices increased the firing rate of presumed noradrenergic neurons (EC_{50}=26 nM) in a concentration-dependent manner. Given i.c.v., senktide (0.5–2 μg) and (MePhe^7)neurokinin B (1–10 μg), another NK_3 receptor agonist, reduced exploratory behaviour in gerbils in a dose-dependent manner (2 μg of senktide producing a 50% reduction of locomotor activity and rearing). *In vivo* microdialysis experiments in urethane-anaesthetized guinea-pigs showed that senktide (2–8 μg i.c.v.) induced a dose-dependent increase in norepinephrine release in the medial prefrontal cortex. The electrophysiological, behavioural and biochemical changes elicited by senktide were concentration- or dose-dependently reduced by SR 142801, the selective non-peptide NK_3 receptor antagonist. In the locus coeruleus slice preparation, complete antagonism of senktide (30 nM) was observed with 50 nM of SR 142801, while injected i.p. (0.1–1 mg/kg) it abolished the senktide-induced norepinephrine release in guinea-pigs. In gerbils, SR 142801 (1–10 mg/kg i.p.) reversed the reduction of exploratory behaviour induced by senktide (1 μg). By contrast, the 100-fold less active enantiomer, SR 142806, did not exert any antagonism in these models. Finally, the reduction of exploratory behaviour in gerbils was found to be reversed by prazosin (0.25–256 μg/kg i.p.) and to some extent by clonidine, drugs known to depress noradrenergic function.

All these experiments strongly support the hypothesis that brain noradrenergic neurons can be activated by stimulation of neurokinin NK_3 receptors. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: locus coeruleus, guinea-pig, gerbil, α-adrenergic receptor, SR 142801, senktide.

Neurokinins, a family of neuropeptides widely distributed in the mammalian central and peripheral nervous system, are involved in various physiological processes such as vasodilation, smooth muscle contraction, bronchoconstriction, salivary secretion, nociception, activation of the immune system and stress (for review see Ref. 24). These biological responses are thought to be mediated through at least three distinct neurokinin receptor subtypes, referred to as neurokinin 1 (NK_1), neurokinin 2 (NK_2), and neurokinin 3 (NK_3)[16] and associated with three different endogenous ligands: substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), respectively. However, these endogenous ligands present a high degree of cross-activity with the neurokinin receptor subtypes,[40] and the use of selective neurokinin agonists and antagonists was required to identify the neurokinin receptor subtype involved in a given biological response.

Several lines of evidence suggest the involvement of NKB in the modulation of central monoaminergic systems. Binding and autoradiographic studies have demonstrated the presence of NK_3 receptors and/or their messenger RNA in various brain areas including the substantia nigra, the raphe nuclei and the locus coeruleus (LC) from various species.[9,39,45,48] Although the NK_3 receptor gene is expressed in human brain, the presence of the receptor protein has not yet been demonstrated, perhaps because binding studies were not performed with the most appropriate ligand.[5,19] Senktide, a potent NK_3 receptor agonist, increases the firing rate of dopaminergic neurons in rat substantia nigra slices.[22] (Pro^7)NKB, another NK_3 receptor agonist, induces the release of (^{3}H)dopamine in matrix-enriched areas of the rat striatum.[47] *In vivo*, injection of neurokinin NK_3 agonists into the rat substantia nigra has been reported to modulate striatal dopamine metabolism[20] and to induce orofacial movements.[23] NkB has also been demonstrated to stimulate the basal and the

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Abbreviations: ACSF, artificial cerebrospinal fluid; HPLC, high-pressure liquid chromatography; LC, locus coeruleus; NK_1, NK_2, NK_3, neurokinin receptor subtypes; NKA, neurokinin A; NKB, neurokinin B; SP, substance P.

evoked release of (^{3}H)serotonin from slices of the rat cerebral cortex,[43] and a serotonin-dependent increase in locomotor activity was observed when senktide was injected into the rat median raphe nucleus.[25,29,30]

In relation to noradrenergic systems, few functional data have been reported to date. Significantly, however, in the guinea-pig LC slice, senktide perfusion induced an increase in firing rate,[27,41] while preliminary experiments in guinea-pig suggest that this peptide enhances Fos-like immunoreactivity in this brain structure and projecting cortical areas.[49] These results are of potential importance since the LC noradrenergic system is implicated in a wide range of physiological and pathological processes, including learning and memory,[13,17] regulation of sleep and arousal,[3,33] anxiety and affective disorders.[28,35]

The aim of the present study was to combine *in vitro* electrophysiological (LC slice preparation) with *in vivo* biochemical (norepinephrine release in the frontal cortex using microdialysis) and behavioural (locomotor activity and rearing) approaches to investigate further the response of noradrenergic systems to NK_3 receptor stimulation. For that purpose, selective agonists for NK_3 receptors [(MePhe^7)NKB and senktide] were used in comparison with NK_1 [(Sar^9, Met(O_2)^{11})SP and septide] and NK_2 [(NLe^{10})-NKA(4–10)] receptor agonists.[36] To characterize the pharmacological responses further, we used specific competitive antagonists for neurokinin receptors. These included SR 142801 (R)-*N*-(1-[3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl]-4-phenylpiperidin-4-yl)-*N*-methylacetamide, the first non-peptide neurokinin NK_3 receptor antagonist, and its 100-fold less active enantiomer, SR 142806,[10] SR 140333, a selective antagonist at NK_1 receptors,[11] and SR 48968, a selective antagonist at NK_2 receptors.[12] SR 142801 displays a species-dependent profile, with 100-fold higher affinities for guinea-pig and gerbil cortex and human NK_3 receptors expressed in CHO (Chinese Hamster Ovary) cells, than for rat cortex NK_3 receptors.[10] Accordingly, electrophysiological and biochemical studies were performed in guinea-pigs, while the gerbil was preferred for psychopharmacological studies because the guinea-pig exhibits a poor behavioural profile.

EXPERIMENTAL PROCEDURES

Animals

Adult Mongolian gerbils (*Meriones unguiculatus*) weighing 60–80 g were used for *in vivo* experiments. The animals were purchased from Janvier Breeding Laboratories (Le Genest-St-Isle, France). Male Hartley guinea-pigs (350–400 g for microdialysis studies and 140–160 g for electrophysiological studies) were purchased from Charles River (St Aubin Les Elbeuf, France). Gerbils were housed six, and guinea-pigs two to a cage. All animals were maintained under a 12-h light/dark cycle (lights on from 7.00 a.m. to 7.00 p.m.) in a room with controlled temperature (21 ± 1°C) and hygrometry (50 ± 10%), and were allowed free access to standard laboratory chow (U.A.R., France) and tap water. All procedures have been approved by the Comité d'Expérimentation Animale (Animal Care and Use Committee) of Sanofi Recherche and were carried out in accordance with French legislation (décret no. 87-848, 19 octobre 1987; and arrêtés, 19 avril 1988) which implemented the European directive 86/609/EEC. In order to perform significant behavioural experiments, a large number of gerbils was required (dose–response curves for agonists and antagonism), thus preventing the use of chronically implanted animals. Therefore, free-hand i.c.v. injections were limited to peptide agonists and special care was taken to avoid any sign of discomfort in the animals.

In vitro *experiments*

Preparation of brain slices. Guinea-pigs were anaesthetized with ketamine (200 mg/kg i.p.) and killed. Slices were prepared as originally described by Henderson.[18] Briefly, the whole brain was gently lifted out of the cranial cavity and placed in cold oxygenated sucrose medium (see below) for 1 min at 4°C to cool it and wash away debris. A block of the brain tissue containing the pons was prepared and glued with cyanoacrylate adhesive (Aron Alpha S-2, Toagosei Chemical Ind., Japan), rostral surface uppermost, on the holder of the Vibratome (Vibroslice, Campden Instruments, U.K.). Then, it was submerged in sucrose medium at 4–6°C and slices were cut at a setting of 350 µm. After a warming-up period of 30 min, a slice containing the LC was placed in a tissue chamber and superfused at a rate of 2 ml/min with artificial cerebrospinal fluid medium (ACSF) saturated with 95% O_2 plus 5% CO_2, and maintained at 35 ± 0.5°C. The ACSF (pH 7.4) was composed of (mM): NaCl 125, KCl 2.5, KH_2PO_4 1.25, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHCO_3$ 26, glucose 10. The sucrose medium used for slice preparation and recovery corresponded to a modified ACSF, where NaCl was replaced by sucrose (250 mM), in order to improve the survival.[2]

Identification of noradrenergic locus coeruleus neurons. When viewed with transmitted light under a binocular microscope, the LC appeared as a relatively translucent crescent-shaped area on the ventrolateral border of the fourth ventricle. Noradrenergic neurons were identified by their electrophysiological properties, and in almost all cases by the application of 10 µM norepinephrine, which blocks their firing. Most neurons fire spontaneously with a constant rate of 0.05–5 Hz and with long-lasting (2 ms) biphasic action potentials. The use of these electrophysiological and pharmacological criteria minimized the possibility of confusion with non-noradrenergic neighbouring neurons.

Recording techniques. After a recovery period of 2 h, extracellular recordings were made by conventional techniques with fibre-filled glass microelectrodes (type GC150F-10, Clark Electromedical Instruments, U.K.), filled with 2.5 M NaCl (d.c. resistances of 30–50 MΩ), using an Axoclamp-2A amplifier (Axon Instruments, U.S.A.). The spikes were amplified, discriminated and computed on line through a CED 1401 interface and IBM-AT computer with suitable software (MRATE 3.03, Cambridge Electronic Design, U.K.). Each experiment was also monitored on an oscilloscope (TDS 420, Tektronix, U.S.A.), and recorded on a thermal pen-recorder (Dash IV, Astro-Med, U.S.A.).

Drugs were applied by perfusion. The ''dead-time'' due to the perfusion system was 20 s. In order to obtain reproducible responses, senktide was perfused for 1 min, with an interval of 20 min between successive applications. This schedule was chosen in order to obtain a reproducible response, i.e. to avoid a tachyphylactic phenomenon such as that observed at shorter intervals during preliminary experiments. SR 142801 and SR 142806 were applied for 1 h and the antagonism effect was evaluated after 20 min, 40 min and 1 h of incubation.

In vivo *pharmacological experiments*

Intracerebroventricular administration of peptide agonists. Microinjections (2 µl, i.c.v.) in gerbils were made free-hand at a depth of 2 mm from the surface of the skull, 2 mm lateral and 2 mm posterior to bregma by means of a 10-µl Hamilton syringe and a 10-mm calibrated needle. Immediately after the i.c.v. injection of septide (0.5–2 µg), (Sar^9, $Met(O_2)^{11}$)SP (1–10 µg), (Nle^{10})NKA(4–10) (1–10 µg), ($MePhe^7$)NKB (1–10 µg), senktide (0.5–2 µg) or vehicle, gerbils were placed in a Digiscan animal activity monitor (Omnitech Electronics, Columbus, OH, U.S.A.). The acrylic cage within the monitor measured 42 × 42 × 30.5 cm. The monitor was equipped with 16 beams 2.54 cm apart from front to back (horizontal), from side to side (horizontal) and from side to side (vertical). The locomotor activity measured during experiments consisted of the total number of beam interruptions on the horizontal sensors during the 20 min following the i.c.v. injection. The number of rears, which consisted of the number of beam interruptions on the vertical sensor, was also recorded.

Inhibition of the senktide-induced reduction of locomotion and rearing. Gerbils were injected i.p. with the selective NK_3 antagonist SR 142801 (1–10 mg/kg) or its enantiomer SR 142806 (10 mg/kg). The animals were also injected i.p. with the selective NK_1 antagonist SR 140333 (1 mg/kg) and the selective NK_2 antagonist SR 48968 (1 mg/kg). At this dose, the two latter compounds have been found to exert complete antagonism on various NK_1 and NK_2 behavioural models.[21,31] To evaluate the implication of noradrenergic systems in the senktide-induced response, gerbils were administered i.p. a selective α_1-adrenergic receptor antagonist, prazosin (0.25–256 µg/kg), a selective α_2-adrenergic receptor agonist, clonidine (0.03 mg/kg), or a β-adrenergic receptor antagonist, propranolol (8 mg/kg). Higher doses of clonidine or propranolol could not be tested because they reduced locomotor activity when given alone. To evaluate further the possible involvement of additional monoaminergic systems in the senktide-induced response, gerbils were administered haloperidol (0.03–0.25 mg/kg i.p.), a mixed D_1/D_2 dopamine receptor antagonist, *S*(–)sulpiride (6.25–12.5 mg/kg i.p.), a D_2 dopamine receptor antagonist, or SR 46349B (0.3–1 mg/kg, *per os*), a serotonin-2 antagonist.[37] Control animals received the vehicle (0.5 ml/100 g body weight). After injection, gerbils were returned to their home cages. Thirty minutes later in the case of an i.p. administration or 60 min later in the case of an oral administration, they were injected i.c.v. with senktide (1 µg) or the corresponding vehicle and placed in the Digiscan cages where locomotor activity or rearing behaviour was recorded for 20 min. These doses and times of pretreatment were chosen according to preliminary experiments in our laboratory.

In vivo *microdialysis experiments*

Surgery, intracerebroventricular pneumatic ejection of senktide and microdialysis procedures. Guinea-pigs were anaesthetized with urethane (1.4 g/kg i.p.) and then mounted in a stereotaxic frame. Their body temperature was monitored by a rectal probe and adjusted (37 ± 1°C) by a homeothermic blanket. The skull and the dura were opened at the levels of the medial prefrontal cortex and the lateral ventricle.

The microdialysis probe (length 3 mm, outer diameter 0.5 mm; CMA 12, Carnegie Medicin AB, Stockholm, Sweden) was implanted in the right medial prefrontal cortex at the following coordinates: 16.6 mm anterior to interaural line, 0.5 mm lateral to the midline, and 5 mm below the cortical surface, according to the atlas of Rapisarda and Bacchelli.[32]

Ejection pipettes consisted of calibrated glass tubes (internal diameter 0.3 mm, 15 mm/µl, Assitent® Ref. 55515, Corning, U.S.A.) and broken back to an external tip diameter of 50 µm. They were implanted into the left lateral ventricle at the following coordinates: 9.8 mm anterior to the interaural line, 2 mm lateral to the midline, and 3.5 mm below the cortical surface, according to the atlas of Rapisarda and Bacchelli.[32] The ejection (2 µl/90 s) was performed by applying air pressure with a 1-ml syringe connected to the non-tapered side of the pipette by tygon tubing.

For control purposes, senktide (10^{-7}–10^{-5} M) was perfused in the medial prefrontal cortex through the microdialysis probe (n = 2–3 animals per concentration group). *In vitro* tests revealed that the probe recovery for senktide was 9%.[44]

The probe was perfused at a constant flow rate of 2 µl/min with ACSF containing (mM): KCl 3, NaCl 125, $CaCl_2$ 1.3, $MgCl_2$ 1.0, $NaHCO_3$ 23, KH_2PO_4 1.5, pH 7.4). A sampling time of 30 min was adopted. After discarding the first 2 h of perfusate, 60-µl samples were collected in Eppendorf microtubes containing 10 µl 0.1 N $HClO_4$, 1 mM EDTA and 4 mM Na metabisulphite. Two baseline samples were collected before pneumatic ejection of senktide. SR 142801 (0.1, 0.3 and 1 mg/kg) or SR 142806 (1 mg/kg) was administered i.p. (5 ml/kg body weight) 30 min before senktide injection. The samples were immediately frozen and stored in a deep freeze (−80°C) no more than four days before assay.

Norepinephrine assay. Norepinephrine levels were assayed by high-pressure liquid chromatography (HPLC) with electrochemical detection. Norepinephrine was separated by a velosep C-18 column (100 × 3.2 mm with 3 µm packing; brownlee columns) using a mobile phase composed of 80 mM sodium phosphate, 100 µM EDTA, 1.2 mM sodium octyl sulfate and 4.3% methanol. The mobile phase was filtered, degassed and adjusted to pH 2.7 with 12 N HCl. The flow rate of the mobile phase was 0.7 ml/min. The HPLC systems consisted of a Wisp 717 delivery system (Waters Associates, CA, U.S.A.) connected to a dual-piston HPLC pump (Waters model 510, CA, U.S.A.) and an external pulse dampener. The analytical system consisted of an electrochemical detector ESA coulochem II equipped with a model 5014 analytical cell (ESA, Chelmsford, MA, U.S.A.).

Materials

Senktide was purchased from Cambridge Research Biomedicals (U.K.) and from Novabiochem (France). ($MePhe^7$)NKB, septide, (Sar^9, $Met(O_2)^{11}$)SP and (NLe^{10})NKA(4–10) were purchased from Novabiochem (France). Clonidine hydrochloride and prazosin hydrochloride were purchased from Pfizer (U.K.). *S*(–)Sulpiride was purchased from RBI (Natick, MA, U.S.A.). Haloperidol, (±)propranolol hydrochloride and norepinephrine hydrochloride were purchased from Sigma (St Louis, MO, U.S.A.). SR 142801 (R)-*N*-(1-[3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl]-4-phenylpiperidin-4-yl)-*N*-methylacetamide, hydrochloride; SR 142806 (S)-*N*-(1-[3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl]-4-phenylpiperidin-4-yl)-*N*-methylacetamide, hydrochloride; SR 140333 (S)-*N*-1-[2-(3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl)-ethyl]-4-phenyl-1-azoniabicyclo(2.2.2)-octane, chloride, SR 48968 (S)-*N*-methyl-N(4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichloro-phenyl)butyl)-benzamide, hydrochloride; and SR 46349B [*trans*, 4-((3*Z*)3-(2-dimethylaminoethyl) oxyimino-3(2-flurophenyl) propen-1-yl)phenol hemifumarate] were synthesized at Sanofi Recherche (Montpellier, France). All other compounds were obtained from Prolabo (France), SDS (France), and Sigma (St Louis, MO, U.S.A.). For *in vitro* studies, drugs were diluted in ACSF from 1 mM stock solutions (in distilled water for peptides and in dimethyl

sulfoxide for SR 142801 and SR 142806). For *in vivo* studies, septide and (Sar^9, $Met(O_2)^{11}$)SP were dissolved in sterile saline. Senktide was dissolved in sterile water. ($MePhe^7$)NKB and (Nle^{10})NKA(4–10) were dissolved in dimethyl sulfoxide at the highest dose and dilutions were made with sterile water. For *in vivo* experiments, SR 142801, SR 142806, SR 140333, SR 48968, SR 46349B and sulpiride were suspended in distilled water with 0.01% Tween 80. Clonidine, prazosin and propranolol were dissolved in distilled water. Haloperidol was dissolved in distilled water containing 0.1% tartaric acid at the highest dose. All concentrations and doses are expressed as the salified form of the compounds, except for the doses of sulpiride and haloperidol which are expressed as the base.

Data analysis

In vitro *experiments*. A dose–response curve was drawn from senktide-induced increases in neuron firing rate. The magnitude of a response is 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. The best-fit for senktide dose–response relationships was calculated using least-square fitting of experimental data to a four-parameter logistic model according to Ratkovsky and Reedy[34] with non-linear RS1 software (BBN Software, Cambridge, MA, U.S.A.). The equation of the curve was written as follows: $A + R_{max}/[1 + \exp(-B \times (\log(C) - EC_{50}))]$ where A was assumed to be null, R_{max} was the amplitude of the response, B was the slope of the curve at EC_{50} abcissa, and (C) the concentration of senktide. Antagonism effects were expressed as a percentage of the senktide control response, and statistical analyses were performed through a one-way analysis of variance (ANOVA) for repeated measures. Comparisons between groups were made using the Student's *t*-test with Bonferroni correction.

In vivo *pharmacological experiments*. All values are expressed as mean ± S.E.M., of eight to 31 animals per group. Results were analysed by the Kruskal–Wallis test and then comparisons between groups were made using the Mann–Whitney test. ID_{50} values were calculated using least-square fitting of experimental data to a four-parameter logistic model[34] with a non-linear curve-fitting RS/1 software. Confidence intervals were calculated with the Newton method.[34]

In vivo *microdialysis experiments*. Norepinephrine levels in serial perfusates were converted to a percentage of the mean value of the two baseline measurements. The area under the curve during the 120 min following the senktide injection was calculated for each treatment group, and statistical analysis was carried out by ANOVA followed by Duncan's test. The ID_{50} for SR 142801 was calculated using least-square fitting of experimental data to a four-parameter logistic model[34] with a non-linear curve-fitting RS/1 software. Confidence intervals were calculated with the Newton method.[34]

RESULTS

Electrophysiological study

A total of 122 spontaneously active neurons with a mean firing rate of 1.5 ± 0.2 Hz was extracellularly recorded in 91 guinea-pig LC slices. Senktide, perfused on these 122 neurons, induced an increase in the firing rate (Fig. 1A illustrates a typical recording) of 85 neurons recorded in 60 slices. The increase induced by senktide was concentration dependent (Fig. 1B) with an EC_{50} of 26 nM (13.9–35.4 nM; 95% confidence limits). The minimal effective concentration was 1 nM, and the maximal response, corresponding to a 4.4 Hz increase in the firing rate frequency, was reached from the concentration of 300 nM. The onset of the response was rapid (less than 1 min) and the duration of the stimulation (4–15 min) related to the concentration, the magnitude of the response being globally independent of the basal activity of the cell (Table 1).

When applied for 1 h at the concentration of 50 nM, SR 142801 inhibited the senktide-induced increase in firing of LC neurons. A typical example on an individual neuron is depicted in Fig. 2A. It shows the progressive action of this compound, reaching its peak after 1 h of bath perfusion. SR 142801, after 20 min of incubation, as shown in Fig. 2B, inhibited the senktide response by 41% ($n = 6$);after 40 min, this inhibition reached a statistically significant value of 73% ($n = 6$), and after 1 h of 93% ($n = 4$). The effect of SR 142801 was less marked at the concentration of 5 nM, but was statistically significant after 1 h (33% inhibition, $n = 5$). The lower concentration of 0.5 nM did not exhibit any effect on the senktide-induced increase in firing rate ($n = 3$). For the two active concentrations, no recovery was observed, even after 2 h of wash-out (data not shown). The inactive enantiomer SR 142806, tested under identical conditions, at the concentration of 50 nM ($n = 3$), did not display any antagonism (Fig. 2C, D). During this study, we never observed any modification of the basal firing rate following application of SR 142801 or SR 142806 (illustrated in Fig. 2B and C).

In vivo *psychopharmacological study*

When gerbils were placed in a novel environment (Digiscan cages) they displayed high levels of locomotor activity and rearing, indicative of exploratory behaviour. Senktide, injected i.c.v., induced an immediate, dose-dependent decrease in locomotor activity, lasting approximately 20–30 min (inset Fig. 3A, B). Senktide induced a significant decrease from 1 μg i.c.v. upwards ($P < 0.01$) and was more potent than ($MePhe^7$)NKB, reducing locomotor activity to 40% of control values at 1 μg i.c.v. when the full peptide

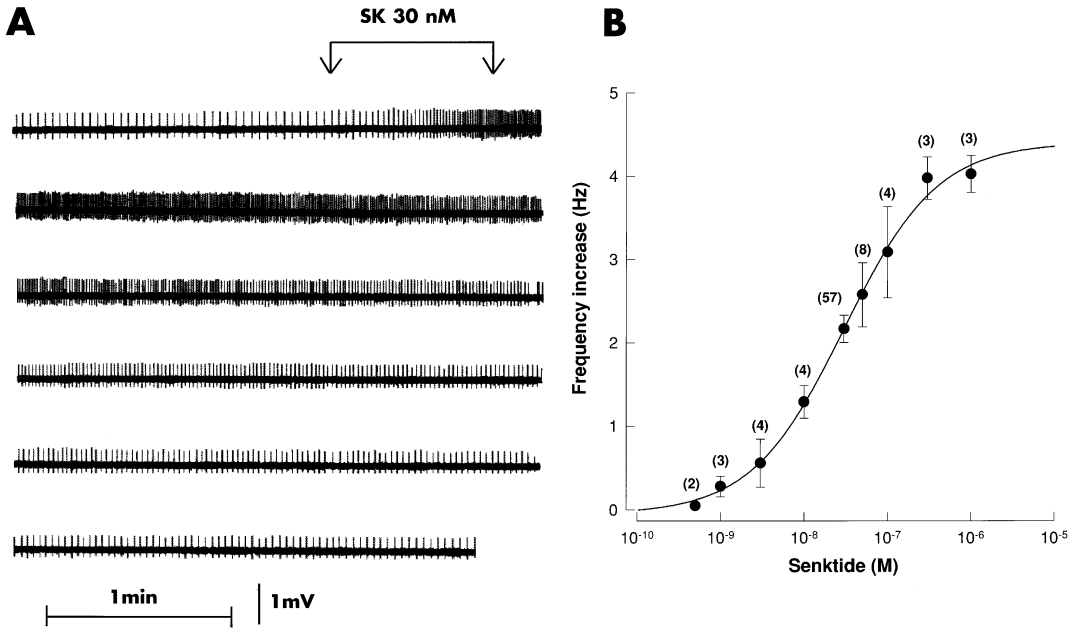

Fig. 1. Senktide-induced increase in firing-rate on neurons of guinea-pig locus coeruleus slices. (A) Typical increase in the firing rate induced by a 1-min perfusion of 30 nM senktide (SK). The spontaneous pre-drug frequency of 0.4 Hz reached a maximal value of about 2 Hz following senktide application. Total recovery was observed after at least 10 min washout. (B) Fitting curve for senktide concentration–response relationship. Values are mean frequency increases ± S.E.M., (*n*) number of cells tested at each concentration.

Table 1. Absence of relationship between rate-increasing effects of senktide and cell basal firing activity

Basal frequency *F* (Hz)	Number of cells	Senktide (30 nM)-induced increase
$0.05 < F < 0.25$	17	2.43 ± 0.33
$0.25 < F < 0.50$	11	1.61 ± 0.16
$0.50 < F < 1.0$	6	1.95 ± 0.32
$1.0 < F < 2.0$	10	1.84 ± 0.25
$2.0 < F < 3.0$	4	2.37 ± 0.67
$3.0 < F < 4.0$	4	3.37 ± 0.90
$F > 4.0$	5	2.32 ± 0.85

Data are the mean ± S.E.M. increase in firing frequency calculated by subtracting the mean frequency occurring during a 100-s control period from the mean frequency occurring during a 100-s senktide response period for a total of 57 of cells subdivided into seven groups according to their basal firing rate.

only reduced locomotor activity to 50% of control values at 10 µg i.c.v. (Fig. 3A). Similarly, senktide and (MePhe^7)NKB reduced the number of rears (Fig. 3B). No other behavioural changes, such as wet dog shakes or face washing as described in rats, were observed during this period. Septide (up to 2 µg, i.c.v.), (Sar^9, Met(O$_2$)^{11})SP (up to 10 µg i.c.v.) and (NLe^{10})NKA(4–10) (up to 10 µg, i.c.v.) failed to induce any significant modification of the locomotor activity of gerbils under the same experimental conditions (data not shown). However, both NK_1 receptor agonists induced vigorous hindpaw tapping.

SR 142801 dose-dependently antagonized the reduction of locomotor activity and the reduction of rearing behaviour induced by senktide (1 µg, i.c.v.) with an ID_{50} of 1.92 mg/kg, i.p. (1.33–3.32 mg/kg; 95% confidence limits) and 6.9 mg/kg, i.p. (4.6–9.1 mg/kg; 95% confidence limits), respectively. By contrast, SR 142806, the inactive enantiomer of SR 142801, failed to alter significantly these behavioural responses at the dose of 10 mg/kg (Fig. 4A, B). No effect on behaviour was observed when SR 142801 was administered alone in the range of doses tested. The selective neurokinin NK_1 receptor antagonist, SR 140333 (1 mg/kg, i.p.), or the selective neurokinin NK_2 receptor antagonist, SR 48968 (1 mg/kg, i.p.), failed to modify the senktide-induced reduction of locomotor activity (Table 2).

The selective α_1-adrenergic receptor antagonist prazosin caused a dose-dependent and complete

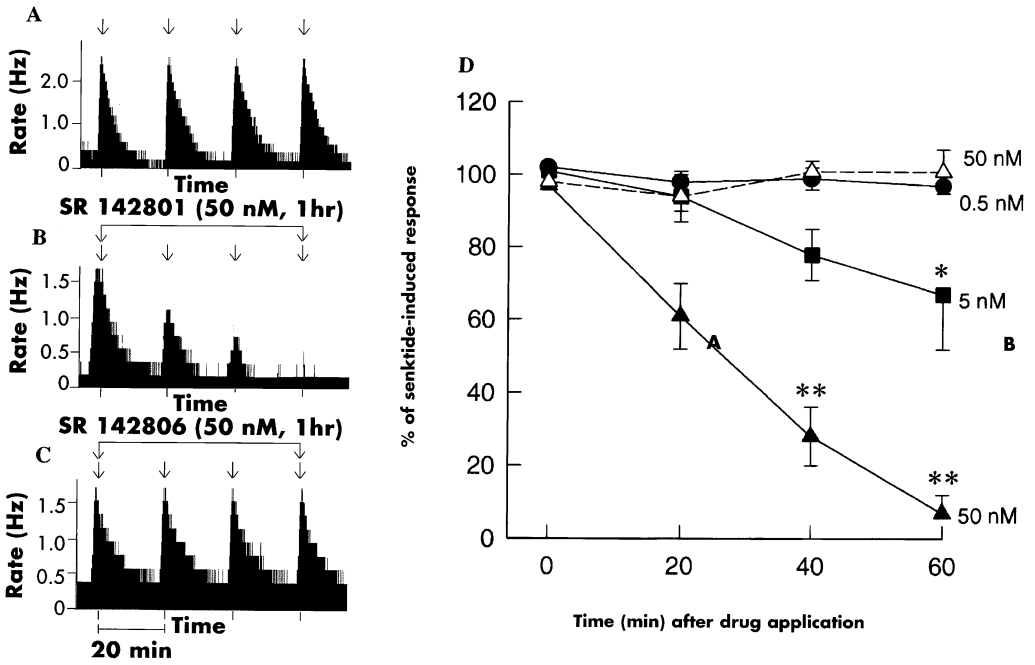

Fig. 2. Reversal by SR 142801 of the senktide-induced response in guinea-pig locus coeruleus neurons. Individual recordings showing (A) reproducibility under control conditions, (B) antagonism by SR 142801 (50 nM), and (C) inactivity of SR 142806 (50 nM). Arrows indicate senktide (30 nM) applications. (D) Concentration and time dependences of the effects of SR 142801 on the response induced by senktide (30 nM). Each point represents the mean ± S.E.M. percentage of the initial control senktide response (n = 3–6 cells). SR 142801: 0.5 nM (●), 5 nM (■), 50 nM (▲); SR 142806: 50 nM (△). *P<0.05, **P<0.01 (Student's paired t-test).

reversal of the reduction of locomotor activity and rearing behaviour induced by senktide (Fig. 5A, B). The ID_{50} for prazosin was 2.54 μg/kg, i.p. (0.08–15.5 μg/kg; 95% confidence limits) and 8.01 μg/kg, i.p. (2.28–32.8 μg/kg; 95% confidence limits), respectively. In addition, this compound, which neither affected spontaneous exploratory behaviour by itself, nor reduced the septide-induced hindpaw tapping in gerbils, was found not to inhibit the (^{125}I)iodohistidyl-(MePhe^7)NKB binding to NK_3 receptors. The selective α_2-adrenergic receptor agonist clonidine caused a slight reversal of the reduction of locomotor activity induced by senktide. The mean locomotor activity (± S.E.M.) recorded after senktide alone or following clonidine (0.03 mg/kg, i.p.) pretreatment was 3169 ± 468 and 5759 ± 650, respectively (P < 0.05). A higher dose of clonidine could not be tested because it reduced locomotor activity when given alone. Pretreatment with a β-adrenergic receptor antagonist, propranolol, failed to reverse the behavioural response induced by senktide. The mean locomotor activity (± S.E.M.) recorded after senktide alone or following propranolol (8 mg/kg, i.p.) pretreatment was 5115 ± 694 and 5767 ± 804, respectively.

The dopamine receptor antagonists haloperidol and sulpiride and the selective serotonin-2 receptor antagonist SR 46349B failed to reverse, and in the case of haloperidol even worsened, the reduction of locomotor activity and rearing behaviour induced by the administration of senktide (1 μg, i.c.v.), as shown in Table 2.

Microdialysis study

Pneumatic ejection of senktide (2, 4 and 8 μg) into the lateral ventricle of the guinea-pig produced a long-lasting, dose-dependent increase in extracellular norepinephrine levels measured in the medial prefrontal cortex (Fig. 6). At the dose of 8 μg, the increase was observed from the first 30-min sample, and a maximal elevation was obtained (+166.0 ± 57.0%) at 60 min. As revealed by examination of the area under the curve (Fig. 6, inset A), senktide induced a significant (P < 0.01) increase in extracellular norepinephrine levels from 2 μg i.c.v. onwards. SR 142801, administered i.p. 30 min before senktide, dose-dependently blocked the effects of senktide (8 μg, i.c.v.). A significant effect was observed from 0.3 mg/kg onwards (P < 0.01) and the ID_{50} for SR 142801 was 0.35 mg/kg (0.23–0.52 mg/kg; 95% confidence limits). By contrast, SR 142806, the (*S*) enantiomer of SR 142801 at the dose of 1 mg/kg, was totally ineffective under the same experimental conditons (Fig. 6, inset B).

Local perfusion of the medial prefrontal cortex by senktide for 60 min did not significantly affect basal extracellular norepinephrine. Maximal increases in

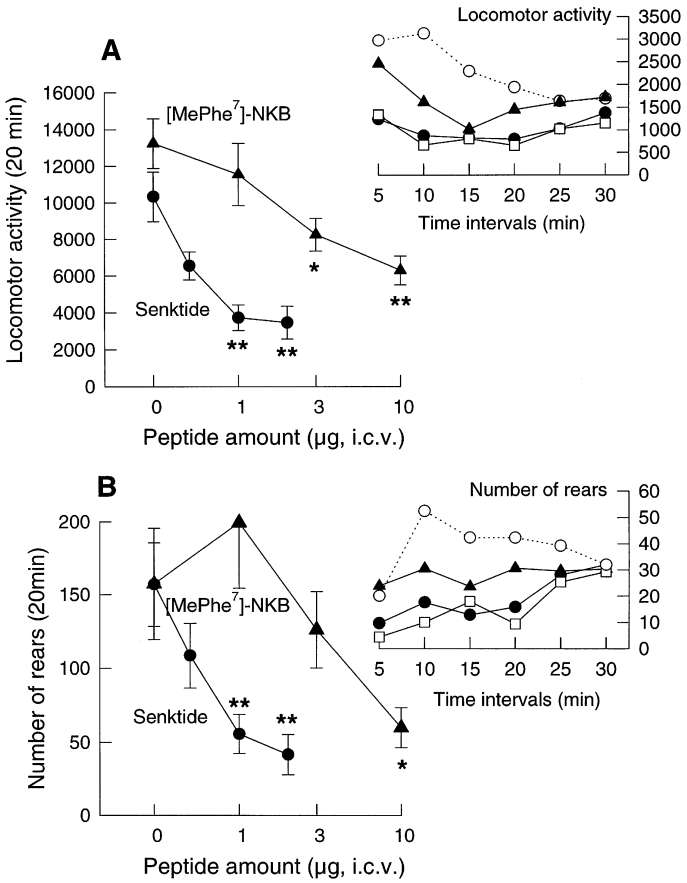

Fig. 3. Effect of NK_3 receptor agonists on exploratory behaviour in gerbils. (A) Reduction of locomotor activity induced by senktide (●) and ($MePhe^7$)NKB (▲) given i.c.v. (B) Reduction of rearing behaviour induced by senktide (●) and ($MePhe^7$)NKB (▲) given i.c.v. Results are mean ± S.E.M. number of beam interruptions on the horizontal or the vertical sensors over the 20-min period following the injection of vehicle or peptide agonists. Significantly different from vehicle controls using Mann–Whitney test: $^{*}P<0.05$, $^{**}P<0.01$. Insets: time-course of the senktide-induced effects by serial 5-min periods. Results are mean number of beam interruptions on the horizontal or the vertical sensors by 5-min periods over the 30-min period following the i.c.v. injection of vehicle (○), or senktide 0.5 µg (▲), 1 µg (●) and 2 µg (□).

cortical norepinephrine were: +25 ± 10% after 0.1 µM (n = 3) and +10 ± 21% after 10 µM (n = 2).

DISCUSSION

Our study yields converging evidence that noradrenergic systems could be modulated by the activation of NK_3 receptors. This was revealed *in vitro* by electrophysiological experiments in guinea-pigs and *in vivo* by behavioural and microdialysis studies in gerbils and guinea-pigs.

We found that application of senktide on guinea-pig LC slices increases cell firing rate in 70% of the cells tested, these neurons having the electrophysiological and pharmacological characteristics of noradrenergic cells. However, a substantial proportion of cells (about 30% of senktide (30 nM)-responsive neurons) was found to display spontaneous rates lower than 0.25 Hz. This unusual low firing might be related to the use of young guinea-pigs or to the inclusion of non-noradrenergic neurons in the sample of cells. The senktide-induced increase in firing rate was concentration dependent, with an EC_{50} of 26 nM. These results are in accordance with those of Seabrook *et al.*,[41] showing that 78% of the cells tested responded to senktide with an EC_{50} of 5 nM for this peptide, and those of McLean *et al.*[27] showing that the application of 1 nM of senktide for about 3 min induced a two-fold increase in firing in guinea-pig LC neurons which was not reversed by a selective NK_1 antagonist. The senktide-induced activation was mediated by the stimulation of NK_3 receptors, since this activation was antagonized in a concentration-related manner by SR 142801, while the inactive enantiomer, SR 142806, did not exert any activity at a concentration for which SR 142801 abolished the senktide response. The onset of the effect of SR 142801 was slow since complete blockade of the senktide response required 1 h of drug (50 nM) application. A delayed, slowly reversible *in vitro* activity, as distinct from that observed *in vivo*, seems to be a typical feature of compounds of this chemical series.[1,11]

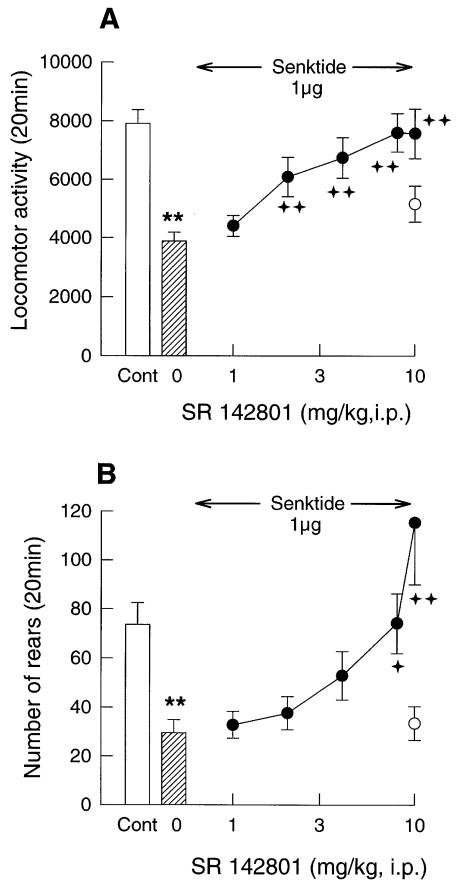

Fig. 4. Effect of the NK_3 receptor antagonist on the behavioural effects induced by senktide (1 µg, i.c.v.). (A) Reversal of senktide-induced reduction of locomotor activity by SR 142801 (●) or SR 142806 (○) given i.p. (B) Reversal of senktide-induced reduction of rearing behaviour by SR 142801 (●) or SR 142806 (○) given i.p. Results are mean ± S.E.M. number of beam interruptions on the horizontal or the vertical sensors over the 20-min period following the injection of vehicle or senktide. Significantly different from vehicle controls, $^{**}P<0.01$; from senktide controls, †$P<0.05$; ††$P<0.01$ using Mann–Whitney test.

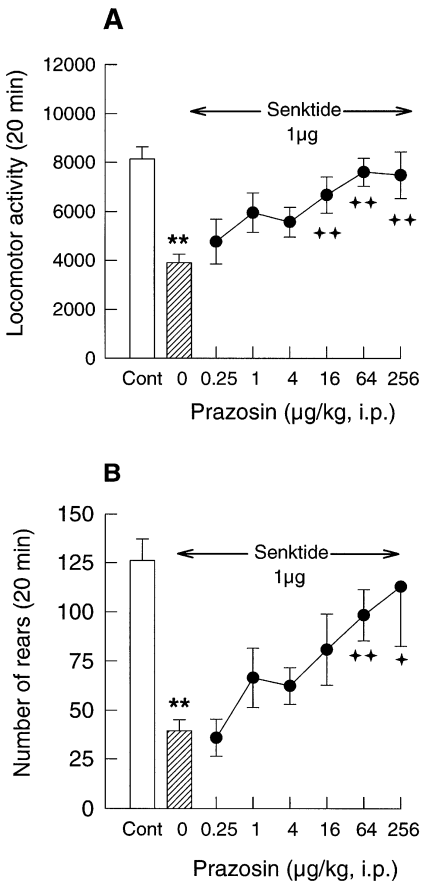

Fig. 5. Effect of prazosin on the reduction of exploratory behaviour induced by senktide (1 µg, i.c.v.). (A) Reversal of senktide-induced reduction of locomotor activity. (B) Reversal of senktide-induced reduction of rearing behaviour. Prazosin was injected i.p. 30 min before the test. Results are mean ± S.E.M. number of beam interruptions over the 20 min on the horizontal sensors or the vertical sensors period following the injection of vehicle or senktide. Significantly different from vehicle controls, $^{**}P$ <0.01; from senktide controls, †$P<0.05$; ††$P<0.01$ using Mann–Whitney test.

Taken together, the electrophysiological effect of senktide and its stereoselective reversal by SR 142801 add strong support to the hypothesis that, in addition to NK_1 receptor activation, as previously demonstrated,[7,26,27] NK_3 receptor stimulation may potently affect the activity of LC noradrenergic neurons.

In vivo experiments in gerbils suggested that the NK_3 receptor agonists senktide and (MePhe^7)NKB injected i.c.v. induced a reduction of exploratory behaviour. Reduction of locomotion and rearing occurred within the first minutes after the placement of the gerbils in their novel environment, animals recovering normal activity within 20–30 min. No other overt modification of behaviour could be observed. By contrast, given i.c.v., selective NK_2 (Nle^{10})NKA(4–10) or NK_1 [(Sar^9, Met(O$_2$)^{11}]SP and septide) receptor agonists were unable to reduce motility in gerbils while, as already reported,[4,38] the latter were found to induce vigorous hindpaw tapping. SR 142801 completely reversed the senktide-induced reduction of both components of exploration, with an ID_{50} very similar to that observed on the turning behaviour induced by intrastriatal injection of senktide.[10] By contrast, SR 142806, the inactive enantiomer, was ineffective, as were SR 140333 and SR 48968. These observations converge to indicate that the behavioural effects induced by senktide were predominantly related to activation of NK_3 receptors.

The senktide-induced modifications of behaviour were antagonized by prazosin but not by propranolol, thus suggesting that NK_3 agonists may reduce exploratory behaviour through the release of norepinephrine acting at central postsynaptic adrenergic α_1 receptors. This hypothesis was supported by the following observations: (i) the reduction of exploratory behaviour was partially reversed by clonidine, which is known to depress noradrenergic function (electrical activity and transmitter release); and (ii) microdialysis

Table 2. Lack of effect of blockade of neurokinin NK_1, NK_2, dopamine and serotonin 2 receptors on senktide-induced reduction of locomotor activity

Treatment		Locomotor activity (20 min)
Control		10,468 ± 555
Senktide	1 μg i.c.v.	4761 ± 454**
	+ SR 140333 1 mg/kg i.p.	5291 ± 446**
	+ SR 48968 1 mg/kg i.p.	4573 ± 667**
Control		8886 ± 377
Senktide	1 μg i.c.v.	4797 ± 460**
	+ haloperidol 0.03 mg/kg i.p.	5560 ± 762**
	+ haloperidol 0.06 mg/kg i.p.	4498 ± 665**
	+ haloperidol 0.12 mg/kg i.p.	2732 ± 328**††
Control		11,185 ± 598
Senktide	1 μg i.c.v.	3786 ± 749**
	+ sulpiride 6.25 mg/kg i.p.	3425 ± 446**
	+ sulpiride 12.5 mg/kg i.p.	2774 ± 427**
Control		9139 ± 803
Senktide	1 μg i.c.v.	3342 ± 368**
	+ SR 46349B 0.3 mg/kg per os	3532 ± 362**
	+ SR 46349B 1 mg/kg per os	2535 ± 229**

Results are mean ± S.E.M. number of beam interruptions over the 20-min period on the horizontal sensors following the injection of vehicle or senktide. Significantly different from vehicle: $^{**}P<0.01$; from senktide: ††$P<0.01$ using Mann–Whitney test.

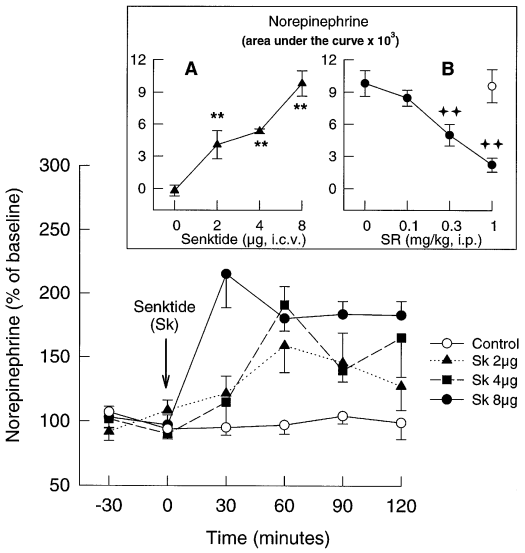

Fig. 6. Effects of senktide (i.c.v.) on extracellular norepinephrine levels measured by microdialysis in the medial prefrontal cortex in urethane-anaesthetized guinea-pigs. Fractional norepinephrine levels are expressed as a percentage of the mean value of the two basal samples. The overall mean basal level of norepinephrine was 7.44 ± 0.6 pg/60 μl per 30 min. Data correspond to the mean ± S.E.M. of four to six animals. Inset A: dose–response curve for senktide on norepinephrine levels after area under curve calculation. Inset B: reversal of senktide-induced increase in norepinephrine levels by SR 142801 (●) or SR142806 (○) given i.p. 30 min before senktide (8 μg/2 μl). Data are area under curve ± S.E.M. for each group for the 120-min period after senktide ejection. $^{**}P<0.01$ versus control group; ††$P<0.01$ versus senktide group using Duncan's test.

experiments performed in the guinea-pig showed that senktide given i.c.v. (at amounts slightly higher that those found to reduce exploratory behaviour in gerbils) induced a sustained and relatively fast-onset increase in norepinephrine release in the medial prefrontal cortex. This effect was abolished stereoselectively by SR 142801 versus SR 142806, thus supporting its mediation by NK_3 receptors.

NK_3 receptor stimulation has been reported to activate directly or indirectly additional monoaminergic systems, including dopaminergic[22,47] and serotoninergic[43] neurons. The failure of dopaminergic receptor antagonists (haloperidol and sulpiride) and SR 46349B, a selective serotonin-2 receptor antagonist, to reverse the reduction of locomotor activity suggested that these monoaminergic systems were not critically involved in the senktide-induced response. NK_1 receptors are present in the LC,[9,39] and NK_1 agonists have been reported to activate noradrenergic cell firing.[27,41] Septide and [Sar^9, Met$(O_2)^{11}$]SP did not produce similar reduction of exploratory behaviour in our study, while inducing vigorous, prazosin-insensitive hindpaw tapping, a behavioural response previously reported.[4,38] In light of these results, one can hypothesize that NK_1 or NK_3 receptor activation, either at the level of the LC or within terminal areas, might involve distinct norepinephrine-containing neuronal populations.

In rodents, increased norepinephrine neuronal activity (transmitter release and/or electrical activity) such as that elicited by a wide range of stimuli including environmental changes, novelty or stressful conditions, has been associated with alertness, behavioural inhibition, and more tentatively with fear or anxiety.[6,14,42,46] Taken together, our findings showing a close association between the level of noradrenergic transmission and behavioural inhibition are compatible with the physiological roles ascribed to noradrenergic systems.

The question arises as to whether a single physiological process associated with NK_3 receptor stimulation can be hypothesized from the electrophysiological, behavioural and biochemical events independently measured after senktide application.

The fact that the behavioural and biochemical changes we observed in gerbils and in anaesthetized guinea-pigs did not exhibit strict parallelism in terms of both doses and time-courses could be accounted for by several procedural factors. They may relate to the animal species, the anaesthesia, the low time constant of microdialysis techniques, or the selection of the cortex as a typical noradrenergic projection area.

The LC is the main source of norepinephrine innervation of the medial prefrontal cortex.[15] Thus, in spite of possible physiological differences in the LC system in young (electrophysiological experiments) vs adult (microdialysis studies) guinea-pigs, the increase in norepinephrine observed in this cortical area could be correlated with a NK_3 receptor-dependent activation of LC neurons such as that found in the slice preparation. However, cortical areas are reported to express NK_3 receptor messenger RNA and to contain NK_3 receptors.[8,45,48] Thus, part of the senktide-enhanced norepinephrine release, as measured in microdialysis experiments or suggested by behavioural studies, can be accounted for by the stimulation of NK_3 receptors, possibly located either on norepinephrine terminals or on neuronal elements involved in the heterologous control of norepinephrine release. Although the number of experiments precluded a firm conclusion, the lack of effect of senktide when applied through the microdialysis probe in the medial prefrontal cortex did not further support this hypothesis.

CONCLUSIONS

The present study yields converging evidence that NK_3 receptor activation potently affects the activity (firing or transmitter release) of brain noradrenergic neurons, despite the procedural heterogeneity between our *in vitro* and *in vivo* approaches and the use of different animal species. The availability of the first non-peptide NK_3 receptor antagonist SR 142801 has considerably improved the further characterization of this heteroregulation.

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