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Biochemical and Pharmacological Activities of SR 142948A, a New Potent Neurotensin Receptor Antagonist

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ABSTRACT

SR 142948A, 2-{[5-(2,6-dimethoxyphenyl)-1-(4-(*N*-(3-dimethylaminopropyl)-*N*-methylcarbamoyl)-2-isopropylphenyl)-1*H*-pyrazole-3-carbonyl]amino}adamantane-2-carboxylic acid, hydrochloride, a new and extremely potent neurotensin (NT) receptor antagonist, has been characterized in comparison with SR 48692. This selective compound possesses nanomolar affinities for NT receptors, recognizes the two binding sites described for the NT receptor and fully displaces [^{3}H]SR 48692 specific binding. SR 142948A antagonizes the classical *in vitro* NT effects, *i.e.*, inositol monophosphate formation in HT 29 cells (IC_{50} = 3.9 nM) or intracellular calcium mobilization in Chinese hamster ovary cells transfected with the human receptor. It dose-dependently (0.04–640 $\times$ 10^{-3} mg/kg p.o.) inhibits the turning behavior induced by unilateral intrastriatal injection of NT in mice, with the biphasic profile previously seen for SR 48692. At 0.1 mg/kg (i.p.), it completely antagonizes NT-evoked acetylcholine release in the rat striatum. In contrast to SR 48692, SR 142948A (p.o.) blocks both hypothermia and analgesia induced by i.c.v. injection of NT (mice and/or rats) but is unable to modify the dopamine release evoked by NT injection into the ventral tegmental area. In summary, SR 142948A retains the properties of the lead compound SR 48692 (no intrinsic agonist activity, oral bioavailability, long duration of action and good brain access), reveals a wider spectrum of activity than SR 48692 (probably due to the inhibition of NT receptor subtypes) and represents an additional tool for further exploration of the therapeutic potential of this class of compounds.

Increased interest has been directed toward the tridecapeptide NT since its discovery and characterization in the brain (Carraway and Leeman, 1973, 1976). However, the actual physiological role of NT has not yet been clearly defined, although this peptide produces a wide range of pharmacological effects in both the periphery and the central nervous system (Kitabgi *et al.*, 1985). When centrally administered, NT elicits alterations in behavior, muscle relaxation, hypothermia and antinociception (Nemeroff and Cain, 1985; Kitabgi, 1989). In fact, anatomical, biochemical and behavioral studies have clearly demonstrated that one of the major actions of NT is to modulate dopaminergic systems (Ervin and Nemeroff, 1988; Phillips *et al.*, 1988; Kasckow and Nemeroff, 1991).

The development of specific NTR antagonists is, therefore, essential for further elucidation of the pathophysiological roles of NT and for development of new drugs active in these pathological conditions. In addition to peptidomimetic antagonists that display partial agonist activities, nonpeptide molecules were recently described, such as the compounds UK-73,093 (Snider *et al.*, 1992), L-734836 (Chakravarty *et al.*, 1993), L-737631 (Naylor *et al.*, 1993) and PD 156425 (Kesten *et al.*, 1994). However, all have weak affinities for human NTRs.

The first member of an original chemical series of potent, selective, nonpeptide NTR antagonists, SR 48692 (2-{[1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazol-3-carbonyl]amino}adamantane-2-carboxylic acid), was described a few years ago (Gully *et al.*, 1993). It inhibits most of the *in vitro* and *in vivo* effects of NT and confirms the modulatory role of NT in the central nervous system, in particular in regulating the activity of DA neurons. However, this compound was unable to antagonize hypothermia and analgesia induced by i.c.v. injection of NT or DA release evoked by NT injection in the rat

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ABBREVIATIONS: ACh, acetylcholine; ANOVA, analysis of variance; BSA, bovine serum albumin; $[Ca^{++}]_i$, intracellular free calcium concentration; CHO, Chinese hamster ovary; DA, dopamine; DMSO, dimethylsulfoxide; IP1, inositol monophosphate; NT, neurotensin; NTR, neurotensin receptor; PBQ, phenyl-*p*-benzoquinone; PBS, phosphate-buffered saline; VTA, ventral tegmental area.

VTA, suggesting the existence of heterogeneous NTRs (Dubuc *et al.*, 1994; Steinberg *et al.*, 1994b).

NT triggers its effects by interacting with specific membrane receptors, as revealed by radioligand binding experiments in a number of tissues and cell lines of animal or human origin (Kitabgi *et al.*, 1985; Kanba *et al.*, 1986). Adult rat and mouse brains have been shown to contain, in addition to the high-affinity NTR, low-affinity levocabastine-sensitive binding sites, whose participation in the effects of NT remains to be determined (Schotte and Laduron, 1987).

High-affinity NTRs (NTR1) have been cloned from rat brain and from the human adenocarcinoma cell line HT 29 (Tanaka *et al.*, 1990; Vita *et al.*, 1993). Recently, the low-affinity NTR (NTR2) has been cloned from adult rat brain (Chalon *et al.*, 1996). These two receptors belong to the family of G protein-coupled receptors activating, in the case of NTR1, the phosphoinositide hydrolysis pathway and subsequently mobilization of intracellular calcium. The intracellular signaling pathway for NTR2 remains to be determined. The distributions of the two sites differ, with the high-affinity binding site predominating in neurons and the levocabastine-sensitive site in glial cells. Nevertheless, low-affinity binding sites have been previously described in some brain areas that are devoid of high-affinity binding sites, *e.g.*, the cortex, the dorsal hippocampus and the thalamus (Kitabgi *et al.*, 1987). NT has K_d values for each site in the nanomolar range. In addition, NT is internalized quickly at 37°C when bound to the high-affinity binding site (Mazella *et al.*, 1991; Hermans *et al.*, 1994) and also probably to the low-affinity binding site (Faure *et al.*, 1994). Taken together, these data lead us to surmise that both NT binding sites play a physiological role. In this study, we report the biochemical and pharmacological properties of a new compound chemically related to SR 48692, *i.e.*, SR 142948A, 2-{[5-(2,6-dimethoxyphenyl)-1-(4-(*N*-(3-dimethylaminopropyl)-*N*-methylcarbamoyl)-2-isopropylphenyl)-1*H*-pyrazole-3-carbonyl]amino}adamantane-2-carboxylic acid, hydrochloride (fig. 1).

Materials and Methods

Animals. Male Sprague-Dawley rats (270–320 g) used for brain membrane preparation for binding studies and for electrophysiological and neurochemical (ACh and DA release) studies, female Swiss albino CD1 mice (25–30 g) used for turning behavior studies and male albino guinea pigs (300–350 g) used for brain membrane preparation for binding studies were obtained from Charles River (St. Aubin les Elbeuf, France). Male OFA rats (130–160 g) used for hypothermia studies and male OFA mice (25–30 g) used for hypothermia and analgesia studies were obtained from Iffa Credo (L'Arbresle, France). All experimental animal protocols have been approved by the Animal Care and Use Committee of Sanofi Recherche.

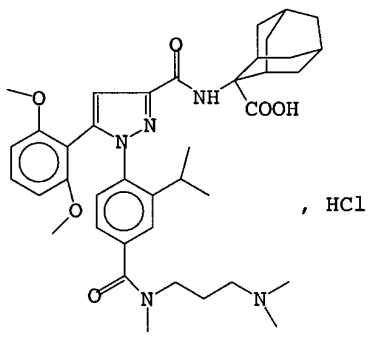

Fig. 1. Chemical structure of SR 142948A.

Chemicals. [^{125}I-Tyr^3]NT (2000 Ci/mmol), [^{3}H]SR 48692 (84 Ci/mmol), [^{3}H]inositol (80 Ci/mmol) and [^{3}H]DA (24 Ci/mmol) were obtained from Amersham (Les Ulis, France). LiCl was purchased from Osi (Oulchy le Château, France) and Dowex-1 AG1-X8 (formate form) was from Bio-Rad (Richmond, CA). NT was purchased from Sigma Chemical Co. (St. Louis, MO) or Neosystem Laboratories (Strasbourg, France) and dissolved in saline. All cell culture-related chemicals were obtained from Gibco (Cergy Pontoise, France). Pargyline, ascorbic acid, desipramine, bacitracin, dithiothreitol, 1,10-orthophenanthroline and PBQ were purchased from Sigma. Levocabastine was a generous gift from Dr. M. Janssen from Janssen Research Foundation (Beerse, Belgium). SR 48692 and SR 142948A (fig. 1) were synthesized at Sanofi Recherche (Montpellier, France). Both compounds were dissolved in DMSO and stored as aliquots at −20°C until the day of experiment, except for the *in vivo* studies, where SR 142948A was solubilized in 0.01% Tween 80 in distilled water (p.o.) or saline (i.p.). All other chemicals were from commercial sources.

Cell culture. CHO cells transfected with the cDNA of the human NTR cloned from HT 29 cells (h-NTR1-CHO cells) were cultured at 37°C in α-modified Eagle's medium without nucleosides, containing 10% fetal calf serum, 4 mM glutamine and 300 μg/ml geneticin (G418), in a humidified incubator under 5% CO_2 in O_2. The HT 29 cell line (obtained from the American Type Culture Collection, Rockville, MD) was cultured under similar conditions in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal calf serum, 4 mM glutamine, 200 IU/ml penicillin and 200 μg/ml streptomycin. For calcium measurements, the h-NTR1-CHO cell culture conditions were slightly modified; the cells were cultured in 25-ml flasks using Dulbecco's modified Eagle's medium containing 2 mM L-glutamine and 10% (v/v) fetal calf serum and supplemented with L-proline, 0.5 mM sodium pyruvate, 0.2% penistreptomycin and 0.06% amphotericin B. One week after seeding, confluent monolayer cultures were washed three times with 3 ml of PBS and harvested by enzymatic dissociation with trypsin. After dilution with PBS, cells were resuspended in the same culture medium at a density of 5×10^4 cells/ml and were plated into 35-mm-diameter, fibronectin-coated, Petri cultures dishes, in the base of which a 16-mm-diameter hole had been cut and replaced by a thin glass coverslip attached with silicon glue.

Membrane homogenate preparation and binding assays. Whole brains or cell pellets were homogenized in 10 volumes (original wet weight/volume) of ice-cold 50 mM Tris-HCl buffer (pH 7.4), for 30 sec, using a Polytron homogenizer (setting 5). After 20 min of centrifugation at 30,000 × *g*, the pellet was washed, centrifuged once again under the same conditions, resuspended in a storage buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% BSA, 40 mg/liter bacitracin, 1 mM 1,10-orthophenanthroline and 5 mM dithiothreitol and stored as aliquots in liquid nitrogen until used. Aliquots of membranes (10, 50, 300 and 500 μg of protein for h-NTR1-CHO cells, HT 29 cells, rat brain and guinea pig brain, respectively) were incubated for 20 min at 20°C in the incubation buffer (0.5-ml final volume) containing appropriate concentrations of [^{125}I-Tyr^3]NT (25–100 pM) and unlabeled drugs. After incubation, the assay medium was diluted with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.1% BSA and 1 mM EDTA, and the mixture was rapidly filtered under reduced vacuum through Whatman GF/B glass fiber filters that had been pretreated with 0.1% polyethylenimine. The filters were washed three times under the same conditions and the radioactivity was measured. Nonspecific binding was determined in the presence of 1 μM unlabeled NT. [^{3}H]SR 48692 binding was determined with a 1-hr incubation at 20°C and filtration on Whatman GF/B glass fiber filters. The nonspecific binding was determined in the presence of 1 μM unlabeled SR 48692. All experiments were performed in triplicate, and data

were expressed as the mean ± S.E.M. of at least three separate determinations. The IC_{50} is the value of ligand that inhibits 50% of the specific binding and was determined by analysis of competition data using an iterative nonlinear regression program (Munson and Rodbard, 1980).

Measurement of inositol phosphate levels. Cells cultured in six-well plates were labeled for 24 hr with 5 μCi/ml [^{3}H]inositol. LiCl (20 mM) was added 15 min before the addition of NT alone or in the presence of various concentrations of SR 48692 or SR 142948A. After 30 min of stimulation, the reaction was stopped by aspiration of the medium and rapid addition of 1 ml of cold methanol/0.1 M HCl (50:50, v/v). The aqueous phase was removed and applied to 1-ml Dowex columns, and IP1 was eluted with 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was quantified by liquid scintillation counting. Results were the mean ± S.E.M. of three determinations performed in triplicate. K_b values were calculated according to the Cheng-Prusoff equation modified by Craig (1993), $K_b = IC_{50}/[1 + ([A]/EC_{50})]$, where the IC_{50} is the value of compound that inhibits 50% of the maximal response observed, EC_{50} is the value of agonist that evokes 50% of the maximal response observed and [A] is the variable concentration of agonist.

Measurement of $[Ca^{++}]_i$. After 1 to 4 days of culture, 3 μl of fura-2/acetoxymethyl ester (Molecular Probes, Interchim, France), taken from a 3 mM stock solution in DMSO, was mixed with 5 μl of Pluronic (200 mg/ml in DMSO) and loaded into the h-NTR1-CHO cells for 25 min at 20°C in the dark, at a final concentration of 3 μM in 2 ml of a buffer (pH 7.4 at 20°C) containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.1% BSA. Cells were washed twice with the same buffer before the addition of 2 ml of buffer without BSA and were further incubated for 60 min at 20°C. The buffer was then removed by aspiration and replaced by 0.2 ml of fresh buffer. The dish was then placed on the plate of the microscope. The bath was continuously perfused (1 ml/min). After a stable basal period, cells were first exposed for 20 sec to 0.1 nM NT (S1). When calcium levels had again reached basal values, the drug to be tested was infused for 40 to 60 sec, and then the cells were exposed for another 20-sec period to a mixture of the drug with 0.1 nM NT or its solvent (S2). At the end of the experiment, internal calibration was performed by manually injecting first a 5 μM final concentration of the Ca^{++} ionophore 4Br-A23187 to assess the maximal fluorescence, then a 16 mM final concentration of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid to assess the minimal fluorescence and, finally, a 10 mM final concentration of $MnCl_2$ to assess the background fluorescence. Fluorescent $[Ca^{++}]_i$ measurements were made at room temperature on single isolated cells. Digital imaging was performed using an IMSTAR (Paris, France) imaging system. Cells were viewed with a Nikon (Tokyo, Japan) Diaphot-TMD microscope, through a Nikon UV-Fluor 40× (NA 1.3) oil-immersion objective. Fura-2 fluorescence was excited alternatively at 350 and 380 nm *via* the microscope epifluorescence port, with illumination provided by a 100-W xenon lamp. Cellular fluorescence was filtered by a 490- to 530-nm bandpass filter (Nikon) and measured with a Darkstar-800 CCD camera (Photonics Sciences, Millham, UK). Images were digitized, *via* an 8-bit ITT VFG 512 × 512 × 4 numerization card, into a PC 486/50 MHz computer and analyzed using IMSTAR STARWISE FLUO software. Ratiometric Ca^{++} images were generated at 2.5-sec intervals. For each cell, $[Ca^{++}]_i$ was averaged from pixels within manually outlined areas. Every cell in the field of the digitized image was quantified, and the 350- and 380-nm fluorescence values generated by the IMSTAR STARWISE FLUO software were transferred into Excel software. The $[Ca^{++}]_i$ was calculated from the 350/380-nm fluorescence ratio as described by Grynkiewicz *et al.* (1985), using *in situ* determined values of the limiting ratios, first in saturating $[Ca^{++}]_i$ (maximal fluorescence) and then in zero $[Ca^{++}]_i$ (minimal fluorescence); the K_d of Ca^{++} for fura-2 was assumed to be 224 nM (Grynkiewicz *et al.*, 1985). The software then calculated the amplitudes of the peaks after each stimulation with NT and the S2/S1 ratio values. Nonresponding cells, where S1 was <2.5 times basal $[Ca^{++}]_i$ values, were discarded.

ACh release in the rat striatum. Rats were anesthetized with urethane (1.4 g/kg i.p.) and mounted in a stereotaxic frame as previously described by Steinberg *et al.* (1995). Their body temperature was maintained at 37 ± 1°C. The skull and the dura were opened to allow implantation of the microdialysis probe into the striatum (at the following coordinates: anterior, 0 mm; lateral, 3 mm; ventral, 6.5 mm; relative to bregma and the dural surfaces). The microdialysis probe (length, 3 mm; outer diameter, 0.5 mm) was perfused, at a constant flow rate of 2 μl/min, with Ringer solution supplemented with 1 μM neostigmine to reduce ACh degradation in the dialysate. ACh levels were estimated in 30-min samples (60 μl) of dialysate by high-performance liquid chromatography with electrochemical detection (Eldec 103). This system includes a trapping precolumn (mobile phase of 35 mM phosphate buffer, pH 8.5) and an immobilized enzyme reactor (BAS MF-6151) that converts ACh to hydrogen peroxide, which is electrochemically detected by a platinum electrode (detection limit, 0.5 pmol/40 μl). Three stable basal ACh values were obtained (90 min) before animals were injected with haloperidol (125 μg/kg i.p.); 30 min later, NT (100 nM) was added to the perfusion medium for 60 min. SR 142948A was administered i.p. 5 min before haloperidol. At the end of the experiment, 10% of animals were randomly sampled for histological study, which confirmed the intrastriatal probe position. The results expressed as calculated areas under the curve during the entire NT perfusion were compared with those of the respective control group. Statistical analyses were performed using nonparametric tests (Mann-Whitney *U* test or Kruskall-Wallis test for multiple comparisons).

DA release in the nucleus accumbens. Rats treated with pargyline and anesthetized 30 min later with urethane were mounted in a stereotaxic frame according to the atlas of Paxinos and Watson (1982). The induced DA overflow was monitored in the nucleus accumbens by electrochemically treated carbon fiber electrodes combined with differential pulse amperometry, as previously described (Steinberg *et al.*, 1994b). Briefly, carbon fiber electrodes were electrochemically treated and implanted in the nucleus accumbens at the following coordinates: 2.2 mm anterior to bregma, 1.5 mm lateral to the midline and 6.5 mm below the cortical surface. The electrodes were connected to a voltamperometric apparatus (Biopulse; SOLEA Tacussel, France), and the catechol oxidation current was monitored every 1 sec with differential pulse amperometry (final potential, +85 mV), as described previously (Suaud-Chagny *et al.*, 1992). Ejection pipettes were filled with NT dissolved in PBS (8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter $Na_2HPO_4 \cdot 2H_2O$, 0.2 g/liter KH_2PO_4, pH 7.4, supplemented with 132 mg/liter $CaCl_2 \cdot 2H_2O$). The pipettes were implanted in the central part of the VTA, with the following coordinates: 5.5 to 5.8 mm anterior to bregma, 0.7 mm lateral to the midline and 8.0 mm below the cortical surface. NT ejection (65 nl) was performed by applying air pressure with a 1-ml syringe connected to the nontapered side of the pipette by Tygon tubing. The ejected volume was determined under the microscope by the movement of the meniscus of the solution in the pipette. NT was applied every 15 min for 90 min.

Turning behavior in mice. Turning behavior induced by unilateral intrastriatal NT injection (10 pg in 1 μl in the presence of 40 mg/liter bacitracin, 5 mM dithiothreitol and 0.1 mM 1,10-orthophenanthroline) was measured in conscious nonrestrained mice (12 animals or multiple per group), according to the method of Poncelet *et al.* (1994a). The number of complete contralateral rotations (away from the injection site) was visually recorded and accumulated over three periods of 2 min (2–4, 6–8 and 9–11 min) after injection. SR 142948A ($0.04–640 \times 10^{-3}$ mg/kg) was administered either i.p. or p.o., 30 min or 60 min before the intrastriatal injection of NT, respectively. In addition, a time-course study was performed at the 2×10^{-3} mg/kg p.o. dose of SR 142948A. Control animals received the corresponding vehicles. Statistical analysis was performed using ANOVA followed by Dunnett's *t* test.

Effects of SR 142948A on population responses of A10 DA neurons in rats. Because the receptor antagonist SR 48692 was recently shown to affect the population response of A10, but not A9, DA cells (V. Santucci, submitted), the effects of SR 142948A were tested only on the former in the present study. After appropriate surgery (Poncelet *et al.*, 1993) in anesthetized male Sprague-Dawley rats (5–10 rats), glass micropipettes (1-μm tips) filled with 0.9% saline or a 0.5 M sodium acetate/2% Pontamine sky blue solution (impedances, 10–15 MΩ at 1000 Hz) were stereotaxically aimed at A10 DA cells. The coordinates of the A10 area were as follows: posterior (to bregma), −5.4 to −6; lateral, 0.4 to 0.8; depth, 7 to 8.5 mm below the cortical surface. After adequate amplification, filtered (200 Hz to 3 kHz) and nonfiltered extracellular action potentials (spikes) were displayed on a digital oscilloscope. Nonfiltered DA cell spikes had a biphasic (positive-negative) waveform (often with a "shouldering" of the positive phase), a slow irregular firing pattern with occasional bursts, a mean ± S.E.M. firing rate of 4.01 ± 0.5 spikes/sec (n = 16) and a spike duration of >2.0 msec, giving a characteristic low-pitch sound on the audio monitor. All of these features were in full agreement with the previously established electrophysiological criteria (Bunney *et al.*, 1991). The number of spontaneously firing neurons per electrode track was determined as previously described (Poncelet *et al.*, 1993), according to the method of Bunney and Grace (1978). SR 142948A (0.010, 0.030 or 0.3 mg/kg i.p., 5 ml/kg) or its vehicle alone was administered 45 to 60 min before initiation of the first electrode track. At the end of some of these experiments, the recording site of the last track was marked by passing a 0.5-μA cathodal current through the electrode for 22 min, to eject sky blue dye. The animals were killed with an i.v. overdose of sodium pentobarbital, the brains were fixed in paraformaldehyde and serial sections were subsequently examined under a light microscope, to locate the dye spot. This confirmed that all of the recordings had been made within the A10 area. Statistical significance for population response was calculated using ANOVA followed by Dunnett's t test.

Retrograde axonal transport of NT in the rat nigrostriatal system. Anesthetized male Sprague Dawley rats were perfused in the right striatum (with the following coordinates: anteroposterior, −0.3; lateral, −3.5; ventral, −5 mm; with respect to bregma) with 30 μg/2 μl thiorphan dissolved in DMSO, according to the method of Steinberg *et al.* (1994a). Ten minutes later, [^{125}I-Tyr^3]NT (0.16 pmol dissolved in saline) was injected in a total volume of 3 μl, in three different areas of the right striatum (with the following coordinates: anteroposterior, +1.7; lateral, −2; ventral, −5 mm; anteroposterior, −0.3; lateral, −4; ventral, −5 mm; anteroposterior, −2.3; lateral, −5; ventral, −5.4 mm; with respect to bregma). After 210 min, the animals were killed by decapitation and each substantia nigra was dissected out. The radioactivity in each structure was measured in a gamma counter. Proteins were assayed according to the method of Bradford (1976). SR 142948A was systemically administered (in suspension with Tween 80 in distilled water) 30 min before the iodinated NT. Statistical comparisons were performed using ANOVA followed by Dunnett's t test.

Hypothermia in mice and rats. In each case, NT injection was performed free-hand in the right lateral ventricle (i.c.v.) of conscious nonrestrained mice (Haley and McCormick, 1957) and rats (Chermat and Simon, 1975). SR 142948A (1–16 mg/kg) was administered p.o. (0.4 ml/20 g and 0.5 ml/100 g b.wt. for mice and rats, respectively) 1 hr before i.c.v. NT (0.2 μg/2 μl and 0.4 μg/2 μl for mice and rats, respectively). Colonic temperature was measured in isolated mice and rats (10 or multiples of 10 animals per group), with a thermocouple probe (Bailey Instruments), just before administration of SR 142948A and 30 min after NT injection. Control animals received the corresponding vehicle. Data are expressed as the mean of variation of temperature between the two measures. Statistical analysis was performed using ANOVA followed by Dunnett's t test.

Antinociception in mice. The effect of NT on PBQ-induced writhings (PBQ test) was measured in animals fasted for 12 hr before the test and isolated throughout the experiment. PBQ dissolved with 5% ethanol in distilled water (2 mg/kg) and maintained at 37°C was administered i.p. (0.2 ml/20 g b.wt.) immediately after i.c.v. injection of NT (2.5 ng/2 μl), performed free-hand, in conscious nonrestrained mice (10 or multiples per group). SR 142948A (0.25–8 mg/kg) was administered p.o. (0.4 ml/20 g b.wt.) 1 hr before NT injection. Writhings were visually recorded between 5 and 15 min after PBQ injection. Control animals received the corresponding vehicle. Statistical analysis was performed using ANOVA followed by Dunnett's t test.

Results

Biochemical profile of SR 142948A in various binding assays. Results were obtained for the inhibition of [^{125}I-Tyr^3]NT specific binding by increasing concentrations of unlabeled NT, SR 48692 and SR 142948A in various animal and human models (IC_{50} values are summarized in table 1). SR 142948A completely displaced the iodinated peptide from the high-affinity binding site present on h-NTR1-CHO cell membranes, with nanomolar affinity (IC_{50} = 1.19 ± 0.15 nM, which determined a K_i value of 1.03 nM) (fig. 2) and a Hill coefficient (n_H) close to unity (1.01 ± 0.01). It also displaced the radioligand from human HT 29 cell membrane preparations (IC_{50} = 0.32 ± 0.08 nM, K_i = 0.28 nM, n_H = 0.91 ± 0.10). In adult rat brain membranes, which are known to possess high- and low-affinity levocabastine-sensitive binding sites, SR 142948A (IC_{50} = 3.96 ± 1.10 nM, n_H = 0.98 ± 0.04) was more potent than SR 48692 (82.0 ± 7.4 nM) (data from Gully *et al,* 1993). Experiments performed in the presence of 10 μM levocabastine completely inhibited the binding of [^{125}I-Tyr^3]NT to the low-affinity binding sites (representing 49% of the population of sites). Under these conditions, SR 142948A exhibited nanomolar affinity for the nonmasked high-affinity binding sites (1.12 ± 0.22 nM, n_H = 1.00 ± 0.13) (fig. 3). In the guinea pig brain binding assay, described as being devoid of levocabastine-sensitive binding sites, both compounds exhibited subnanomolar affinities.

Competition studies performed on guinea pig brain membranes with [^{3}H]SR 48692 as the radioligand revealed that SR 142948A completely displaced the specific binding of this radiolabeled antagonist with a higher potency, compared with unlabeled SR 48692 (IC_{50} = 0.30 ± 0.05 nM, K_i = 0.17 nM and n_H = 0.74 for SR 142948A, compared with IC_{50} = 6.0 ± 1.2 nM, K_i = 3.9 nM and n_H = 0.92 for SR 48692) (table 1; fig. 4). Like the lead compound, this new molecule was selective for the NTR, as revealed by the lack of inhibition, at

TABLE 1

IC_{50} values for the inhibition of specific [^{125}I-Tyr^3]NT binding and [^{3}H]SR 48692 binding in various membrane preparations

Each value represents the mean ± S.E.M. from at least three separate experiments performed in triplicate.

Binding Assay	IC_{50} (*nM*)		
	NT	SR 142948A	SR 48692
[^{125}I-Tyr^3]NT binding			
h-NTR1-CHO cells	0.47 ± 0.07	1.19 ± 0.15	19.3 ± 4.9
HT 29 cells	0.26 ± 0.04[a]	0.32 ± 0.08	30.3 ± 1.5[a]
Adult rat brain	3.20 ± 0.50[a]	3.96 ± 1.12	82.0 ± 7.4[a]
Adult guinea pig brain	2.80 ± 1.60[a]	0.37 ± 0.01	0.99 ± 0.14[a]
[^{3}H]SR 48692 binding			
Adult guinea pig brain	102 ± 13	0.30 ± 0.05	6.0 ± 1.2

[a] Results were previously reported (Gully *et al.*, 1993) and obtained under identical conditions.

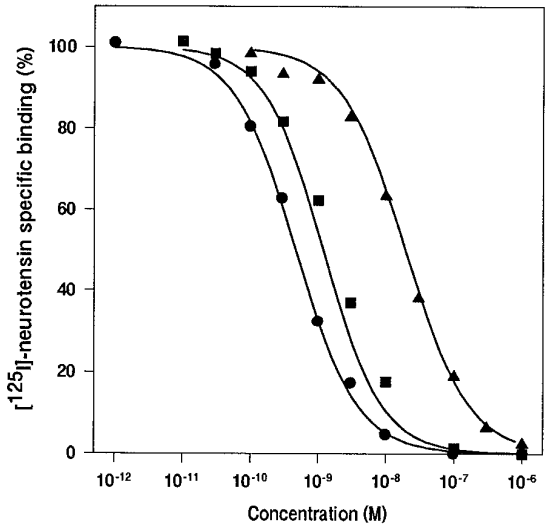

Fig. 2. Inhibition of the specific binding of [^{125}I-Tyr^3]NT to h-NTR1-CHO cell membranes by increasing concentrations of NT (●), SR 142948A (■) and SR 48692 (▲). Reported values are the means of three independent experiments performed in triplicate, and variations did not exceed 5%.

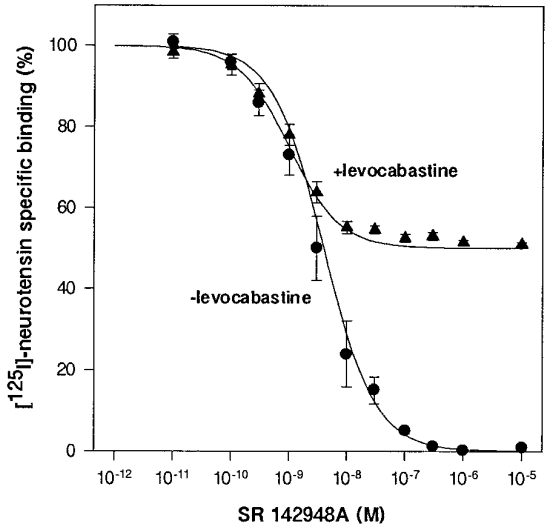

Fig. 3. Inhibition of [^{125}I-Tyr^3]NT specific binding to adult rat brain membranes by SR 142948A, in the absence (●, 100% of sites, n = 3) and in the presence (▲, 51% of sites, n = 5) of 10 μM levocabastine. Results are mean ± S.E.M. values of three to five experiments performed in triplicate.

concentrations up to 1 μM, of a large variety of receptors for neurotransmitters, peptides and hormones (data not shown).

Antagonism by SR 142948A of IP1 production and intracellular calcium mobilization stimulated by NT in h-NTR1-CHO cells. NT stimulated IP1 accumulation in HT 29 cells with an EC_{50} value of 3.5 ± 0.5 nM (basal IP1 level, 31.5 ± 2.4 pmol/10^6 cells; maximal level, 80.3 ± 7.4 pmol/10^6 cells) (data not shown). Figure 5A illustrates the effect of SR 142948A on IP1 formation induced by various concentrations of NT in HT 29 cells. SR 142948A concentration-dependently antagonized the NT-induced response, with IC_{50} values of 3.9 ± 0.07 nM, 11 ± 4.3 nM and 53 ± 3.1 nM for 10, 100 and 1000 nM NT, respectively. The K_b value calculated according to the Cheng-Prusoff equation (see "Materials and Methods") was 0.52 ± 0.25 nM. Similarly, SR

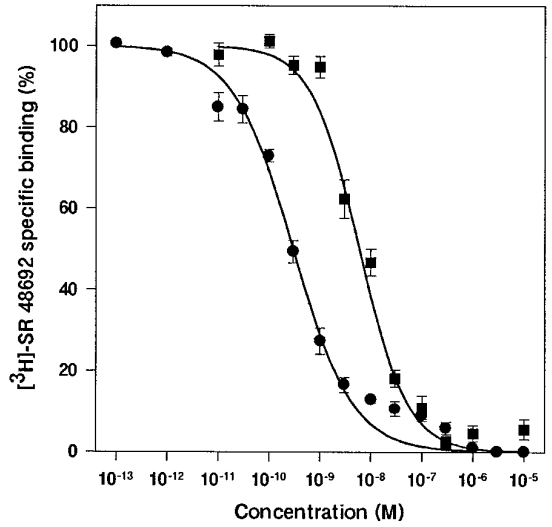

Fig. 4. Inhibition by SR 142948A (●) and SR 48692 (■) of [^{3}H]SR 48692 specific binding to guinea pig brain membranes. Results are mean ± S.E.M. values of at least three experiments performed in triplicate.

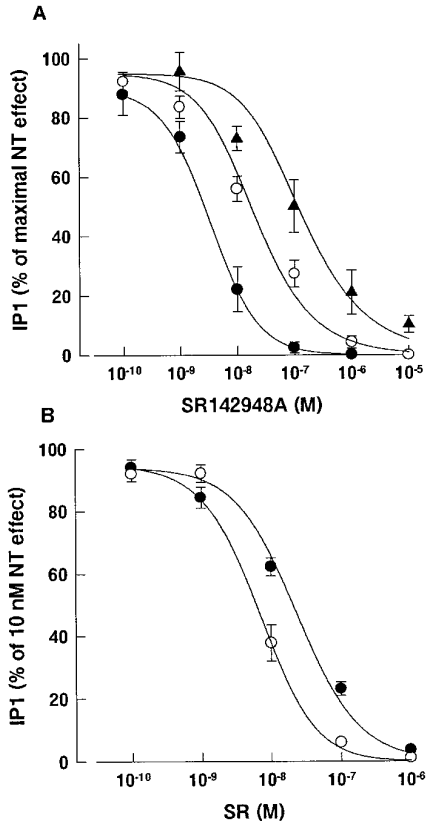

Fig. 5. NT-induced IP1 formation in cultured cells. A, effects of SR 142948A on IP1 formation induced by increasing concentrations of NT [10 (●), 100 (○) and 1000 nM (▲)] in HT 29 cells. The results are expressed as a percentage of maximal NT response. Each point represents the mean ± S.E.M. of triplicate determinations. B, antagonism by SR 142948A (○) and SR 48692 (●) of NT-induced IP1 formation in h-NTR1-CHO cells. The results are expressed as a percentage of the effect of 10 nM NT. Each point represents the mean ± S.E.M. of three independent experiments performed in triplicate.

142948A inhibited IP1 formation induced by 10 nM NT in h-NTR1-CHO cells (fig. 5B) with an IC_{50} value of 9.7 ± 1.7 nM, whereas SR 48692 was approximately 3-fold less effi-

cient in inhibiting this response (IC_{50} value of 22.9 ± 3.2 nM). In all of these experiments, SR 142948A alone (up to 1 μM concentration) did not affect the basal level of IP1 (data not shown).

The results of calcium measurements in h-NTR1-CHO cells perfused twice with NT in the absence of an antagonist showed (fig. 6) that the S2/S1 ratio was close to unity (1.12 ± 0.07, n = 68 cells). When the second stimulation occurred in the presence of 10 nM SR 48692, the S2/S1 ratio was reduced by approximately 50% (S2/S1 = 0.51 ± 0.03, n = 54 cells), whereas the same concentration of SR 142948A abolished the ability of NT to increase free $[Ca^{++}]_i$ (S2/S1 = 0.09 ± 0.02, n = 76 cells). At a concentration of 1 nM, SR 142948A inhibited the second peak of NT by >65% (S2/S1 = 0.35 ± 0.03, n = 30 cells), whereas SR 48692 was inactive (S2/S1 = 0.93 ± 0.05, n = 44 cells) (data not shown).

Neuropharmacological profile of SR 142948A. The *in vivo* efficacy of SR 142948A was determined in the model of turning behavior induced by injection of exogenous NT. An average of 12 contralateral rotations per mouse was induced by 10 pg of NT injected unilaterally in the striatum. Figure 7 shows that, like SR 48692 (Gully *et al.*, 1993; Poncelet *et al.*, 1994a), SR 142948A significantly reduced NT-induced turning with a complex dose-response relationship (antagonism followed by disappearance of the antagonism). Inhibition occurred dose-dependently between 0.04 and 2.5 × 10^{-3} mg/kg whatever the route of administration [i.p. or p.o.; linear regressions with $F(1,146)$ = 31.4, $P < .001$, and $F(1,127)$ = 30.7, $P < .001$, respectively]. The respective ID_{50} values were 0.14 × 10^{-3} mg/kg for p.o. administration (95% confidence limits, 0.02–0.23 × 10^{-3} mg/kg) and 0.13 × 10^{-3} mg/kg for i.p. injection (95% confidence limits, 0.05–0.32 × 10^{-3} mg/kg). In both cases, the disappearance of the antagonism occurred at doses above 2.5 × 10^{-3} mg/kg. Administration of the D_2 receptor blocker spiroperidol (0.03 mg/kg i.p.) abolished the reinstatement of turning in animals treated with a high dose of SR 142948A [SR 142948A (640 × 10^{-3} mg/kg, p.o.), 12.7 ± 2.0 rotations; SR 142948A (640 × 10^{-3} mg/kg, p.o.) plus spiroperidol, 2.0 ± 0.7 rotations; $P < .05$, Dunnett's *t* test], as was previously found with SR 48692 (Poncelet *et al.*, 1994b). With SR 142948A, the range of antagonistic doses was larger than for SR 48692; former compound was also more potent. The time-course study performed with p.o. administration of 2 × 10^{-3} mg/kg SR 142948A (fig. 8) revealed that the compound produced a nonsignificant effect (−37.5%) 30 min after its administration, with maximal and significant antagonism being observed between 1 and 2 hr after its administration (−67.5% and −64% at 1 and 2 hr, respectively). No significant antagonism of rotations by SR 142948A was observed 8 hr after administration of this compound.

Local perfusion of NT (100 nM) by microdialysis for 60 min did not significantly affect striatal ACh levels, compared with

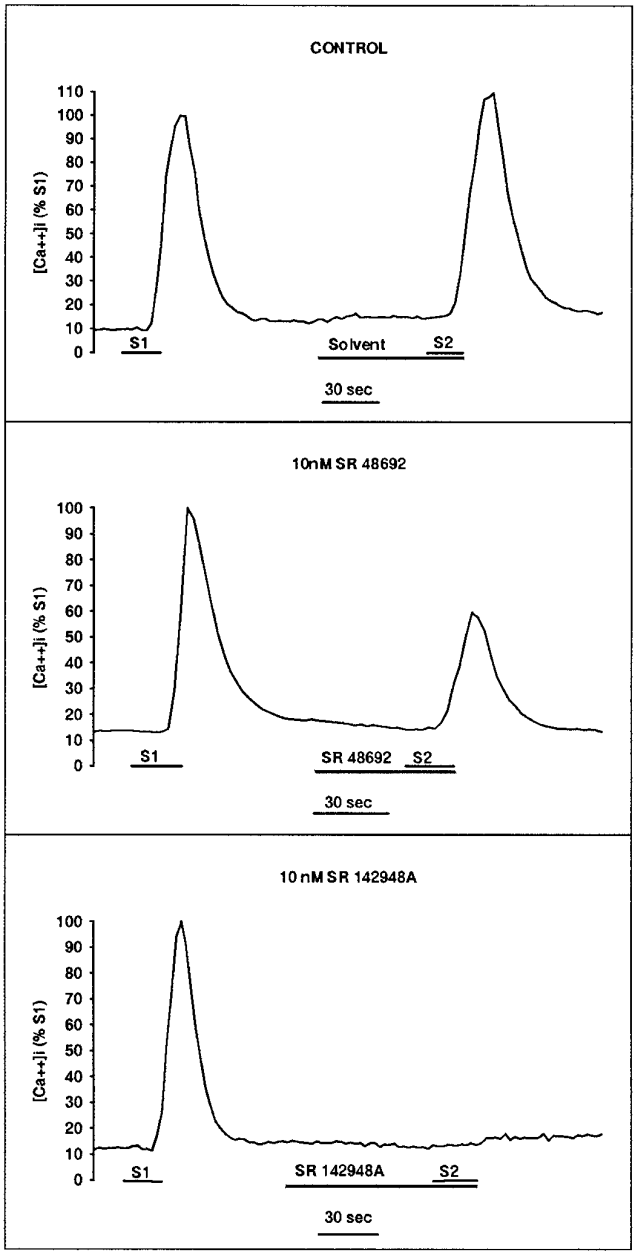

Fig. 6. Effects of 10 nM SR 48692 and of 10 nM SR 142948A on NT-induced $[Ca^{++}]_i$ increases in h-NTR1-CHO cells. S1 and S2, 20-sec infusions with 0.1 nM NT. In the control group, basal $[Ca^{++}]_i$ before S1 was 79 ± 5 nM (n = 68).

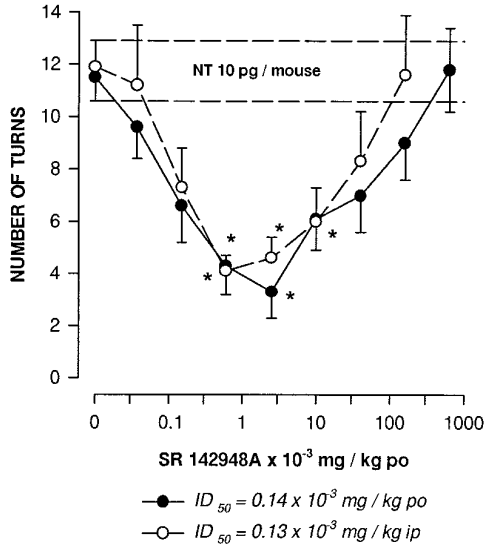

Fig. 7. Effect of SR 142948A administered either i.p. (○) or p.o. (●), 30 or 60 min, respectively, before intrastriatal injection of NT (10 pg/mouse). Data are the mean ± S.E.M. of the number of contralateral turns in 6 min (Dunnett's *t* test, *$P < .05$ *vs.* control).

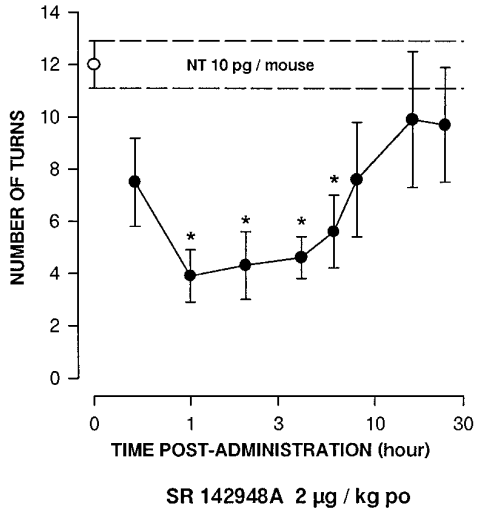

Fig. 8. Time-course study of the inhibition by SR 142948A (2×10^{-3} mg/kg, p.o. administration) of the turning behavior induced by intrastriatal injection of NT (10 pg/mouse). Data are the mean ± S.E.M. of the number of contralateral turns in 6 min (Dunnett's *t* test, *P < .05 *vs.* control).

base-line levels (100 ± 16% at 60 min). However, NT (100 nM) applied locally under D_2 receptor blockade by haloperidol (0.125 mg/kg i.p.) increased ACh levels, with a maximal elevation being observed at 60 min (175 ± 12%). When haloperidol was administered alone, ACh levels were only slightly increased to 125 ± 10%. As shown in figure 9, SR 142948A dose-dependently (0.003–0.1 mg/kg i.p.) prevented the enhancement of ACh release produced by NT. SR 142948A (0.1 mg/kg) alone did not modify basal ACh release (data not shown). Figure 10 illustrates the dose-dependent, significant increase in population response of A10 DA cells after acute i.p. injection of SR 142948A (0.01, 0.03 and 0.3 mg/kg).

When iodinated NT was injected in the right striatum of rats just after thiorphan injection (0.03 mg, as an inhibitor of

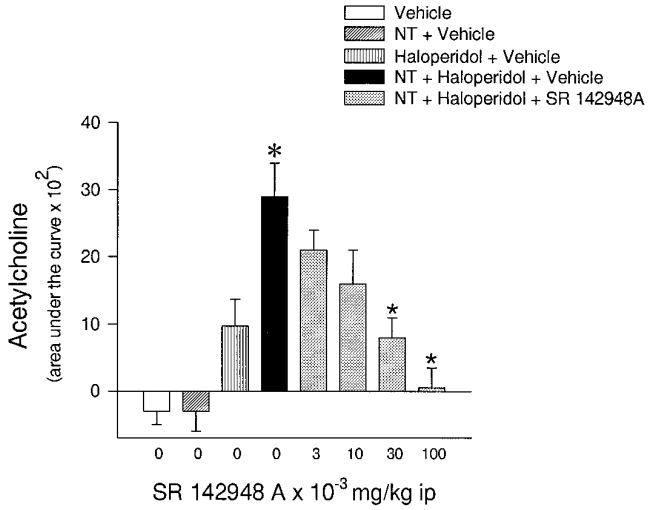

Fig. 9. Reversal of NT (100 nM)-induced increases in ACh levels by SR 142948A given i.p. 5 min before haloperidol (0.125 mg/kg i.p.). Data are the area under the curve ± S.E.M. values for each group for the 60-min period of NT perfusion (small asterisk, P < .05 *vs.* NT plus haloperidol group, using the Kruskall Wallis test; large asterisk, P < .05 *vs.* haloperidol group, using the Mann-Whitney *U* test).

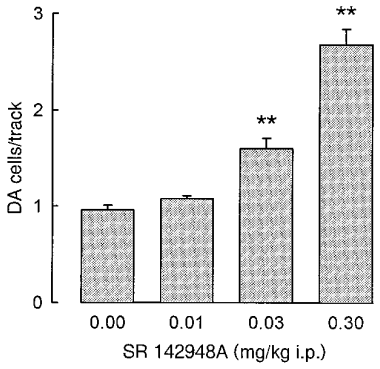

Fig. 10. Effects of single administration of SR 142948A on population responses of A10 DA neurons expressed as the mean ± S.E.M. of the number of spontaneously active cells/track (ordinate). Doses (i.p. injected) are indicated on the abscissa. Control (0.0 mg/kg) value was 0.96 ± 0.05 (n = 10 rats). Drug values were calculated from n = 5 rats/dose (Dunnett's *t* test, **P < .01 *vs.* control).

NT degradation), radioactivity accumulated as a function of time in the ipsilateral substantia nigra (Castel *et al.*, 1994; Steinberg *et al.*, 1994a). Administered i.p. 30 min before the striatal injection of radiolabeled NT, SR 142948A dose-dependently (5–20 mg/kg) and partially reduced (50%, with the dose of 40 mg/kg producing no further inhibition) the retrograde axonal transport of the peptide measured 3.5 hr after NT injection (fig. 11).

NT injected i.c.v. at the dose of 0.4 µg caused a significant, although moderate, decrease (−1.4°C) in body temperature in rats. More marked hypothermia (−3.3°C) was obtained in mice after i.c.v. NT (0.2 µg). SR 142948A partially but significantly blocked NT-induced hypothermia (53% at 2 mg/kg p.o. in rats and 54% at 4 mg/kg p.o. in mice) (fig. 12). The dose-response relationship of SR 142948A was bell-shaped, with disappearance of the effect in both species at doses above 4 mg/kg. In basal conditions, the body temperature was unaffected by SR 142948A treatment (data not shown).

Figure 13 shows that the number of stretches induced by i.p. PBQ injection was significantly reduced by i.c.v. injection of NT (2.5 ng). SR 142948A completely inhibited the antinociceptive effect induced by NT, with a maximum effect at 4 mg/kg and disappearance of this effect at doses of SR 142948A higher than 4 mg/kg. Administration of a D_2 recep-

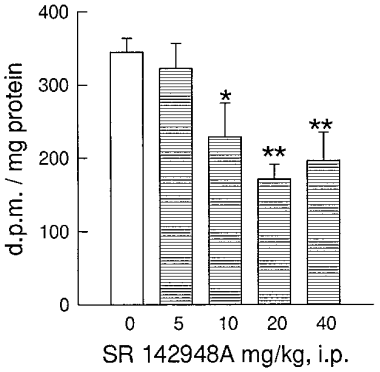

Fig. 11. Accumulation of radioactivity measured in the ipsilateral substantia nigra 3.5 hr after the injection of 0.16 pmol of [^{125}I-Tyr^3]NT into the right striatum and effects of SR 142948A given i.p. 30 min before NT injection. Results are expressed as the mean ± S.E.M. (n = 4–6 rats/group, except for the vehicle-treated group, with n = 14) (Dunnett's *t* test, *P < .05, **P < .01, compared with vehicle-treated group).

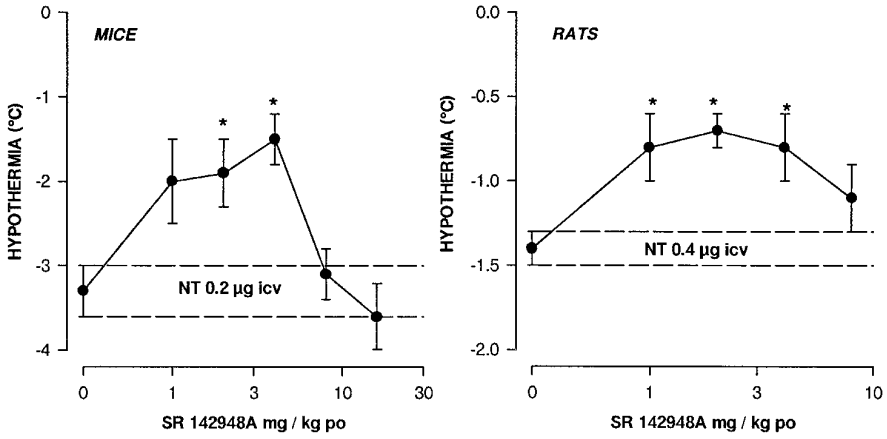

Fig. 12. Effects of increasing doses of SR 142948A p.o. on the hypothermia induced by i.c.v. injection of NT in mice (left) and rats (right). The results are expressed as the mean ± S.E.M. of variations of body temperature (Dunnett's *t* test, *P < .05, compared with vehicle-treated group).

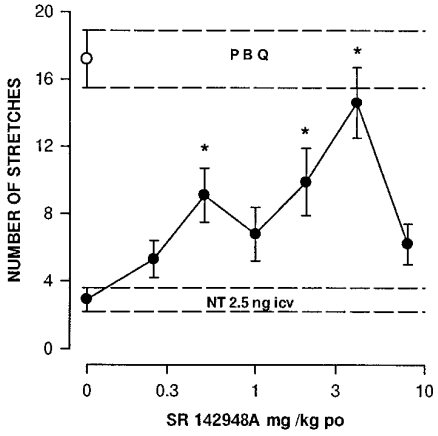

Fig. 13. Effects of increasing doses of SR 142948A p.o. on the antinociceptive effect induced by i.c.v. injection of NT in the PBQ writhing test performed in mice. The number of body stretches was counted in mice after i.p. injection of PBQ. The results were expressed as the mean ± S.E.M. of body stretches (Dunnett's *t* test, *P < .05 *vs.* control).

tor blocker (spiroperidol, 0.1 mg/kg i.p.) did not significantly modify hypothermia or analgesia induced by NT. Spiroperidol (0.1 mg/kg i.p.) prevented the disappearance of the antagonistic effects of SR 142948A at doses above 4 mg/kg p.o. [for example, for hypothermia: SR 142948A (16 mg/kg p.o.), −3.14 ± 0.44°C; SR 142948A (16 mg/kg p.o.) plus spiroperidol, −0.92 ± 0.28°C; for analgesia: SR 142948A (16 mg/kg p.o.), 2.8 ± 1.0 stretches; SR 142948A (16 mg/kg p.o.) plus spiroperidol, 8.2 ± 3.0 stretches].

Ejection of NT (100 nM, 65 nl) every 15 min into the VTA induced highly reproducible short-lasting increases in DA overflow in the nucleus accumbens, as measured by the amplitude of the evoked changes in oxidation current (from 65 to 148 mm, depending on the animal in the control groups, n = 4). SR 142948A (0.1, 1 and 10 mg/kg i.p.) administered after the third NT control ejection did not induce a significant change in NT-induced DA efflux (maximal changes: 0.1 mg/kg, −14 ± 9%, n = 5; 1 mg/kg, +1 ± 3%, n = 3; 10 mg/kg, −16 ± 16%, n = 3).

Discussion

This study describes the biochemical and pharmacological properties of SR 142948A, a novel synthetic NTR antagonist. Like SR 48692, the first antagonist described, this compound belongs to the pyrazole family. It differs from the lead molecule by the replacement of the chloroquinoline substituent in position 1 of the pyrazole by a bisubstituted phenyl ring.

SR 142948A completely inhibits [^{125}I-Tyr^3]NT specific binding to the human high-affinity NTR cloned from the HT 29 cell line and stably expressed in CHO cells (h-NTR1-CHO), with nanomolar affinity close to that of NT (table 1). This high affinity was confirmed in binding assays performed using human HT 29 cell membrane preparations. Binding assays using adult rat brain membranes in the presence or absence of levocabastine (which completely occludes the low-affinity binding site present in this species) suggest that SR 142948A exhibits identical nanomolar affinities for both binding sites (fig. 3). In contrast, in this model SR 48692 has a higher affinity for the high-affinity site than for the low-affinity binding site, as demonstrated by the shift in IC_{50} values obtained in experiments performed in the presence or absence of 10 μM levocabastine (Gully *et al.*, 1993). These data are confirmed by preliminary results obtained in binding assays performed with [^{125}I-Tyr^3]NT using COS-3 cells transiently transfected with the recently cloned levocabastine-sensitive NTR (NTR2). These data give IC_{50} values of 1.8 nM for SR 142948A and 22.5 nM for SR 48692 (data not shown; SR 48692 results from Chalon *et al.*, 1996). However, with guinea pig brain membrane preparations that do not express levocabastine-sensitive binding sites, no difference between the two compounds is observed and both IC_{50} values are subnanomolar, suggesting interspecies differences, as already reported by Cusack *et al.* (1995). The new derivative described here, SR 142948A, recognizes all NTRs with nanomolar affinity close to that of the natural ligand. Like numerous nonpeptide radiolabeled ligands specific for neuropeptide receptors, the tritiated form of SR 48692 recognizes a greater number of NT binding sites than does iodinated NT. SR 142948A completely displaces [^{3}H]SR 48692 specific binding to guinea pig brain membranes, with a higher affinity than that of the unlabeled ligand. These binding sites, characterized by a high affinity for antagonists and low affinity for NT, have the same distribution as the high-affinity NT sites (Betancur *et al.*, 1995; Labbé-Juillié *et al.*, 1995), suggesting the existence of either distinct binding sites for the two compounds or different affinity states for the same ligand recognition site on the NTR. Tested at a concentration of 1 μM in a large variety of binding assays for neurotransmitters, peptides and hormones, SR 142948A appears to be highly selective for the NTR, in a manner comparable to that of SR 48692. To summarize these results, SR 142948A has

the characteristics of an extremely potent (its nanomolar affinity is close to the affinity of the natural ligand in numerous binding models) and selective ligand for human NTRs and is unable to discriminate between high- and low-affinity NT binding sites described as levocabastine-sensitive binding sites in mice and rats.

The binding of NT to its receptor induces an increase in turnover of phosphatidylinositol, which acts as the second messenger for calcium mobilization. Incubation of HT 29 cells with NT in the presence of lithium (an inhibitor of the inositol 1-monophosphatase activity) for a period of time corresponding to binding equilibrium (30 min) produces rapid and transient formation of inositol trisphosphate and inositol bisphosphate and a sustained increase in IP1 levels, as previously shown in the neuroblastoma cell line N1E115 by Amar *et al.* (1986). It was previously demonstrated that SR 48692 is a competitive inhibitor of this NT effect (pA_2 values of 8.7) (Oury-Donat *et al.*, 1995), with lower efficiency than SR 142948A. These results are consistent with binding data obtained using HT 29 cells, which possess only an homogeneous population of high-affinity binding sites.

The activation of phospholipase C by the peptide and the resulting inositol trisphosphate generation lead to intracellular Ca^{++} mobilization. This cascade of events has been verified in HT 29 cells (Bozou *et al.*, 1989), and SR 48692 was shown to behave as a competitive nanomolar antagonist (Gully *et al.*, 1993). In the h-NTR1-CHO cell model, SR 142948A completely antagonizes the NT-induced rise in $[Ca^{++}]_i$, with efficacy comparable to that described for inositol phosphate turnover. In these *in vitro* studies, SR 142948A acts as a competitive antagonist devoid of agonist properties, with an efficacy closely related to the relative binding affinities.

In the striatum, where NT binding sites are partly located on DA terminals, it has been demonstrated that NT facilitates the release of both labeled and endogenous DA evoked by K^+ depolarization in striatal slices. SR 48692 was previously described as inhibiting, in a concentration-dependent manner, the NT-induced enhancement of DA efflux in guinea pig striatal slices ($IC_{50} = 0.35 \pm 0.12$ nM) (Gully *et al.*, 1993), and the same antagonistic property for SR 142948A has been observed ($IC_{50} = 0.27 \pm 0.03$ nM) (M. Heaulme, in preparation).

The ability of SR 142948A to antagonize turning behavior induced by unilateral intrastriatal injection of NT in mice follows a complex dose-response relationship similar to that described for SR 48692, *i.e.*, antagonism followed by reinstatement of rotations (Poncelet *et al.*, 1994a). As found for SR 48692 (Poncelet *et al.*, 1994a), this latter phase was not observed in spiroperidol-treated mice, suggesting that reinstatement of rotations is dependent on DA D_2 regulatory mechanisms. The first part of the U-shape dose-response curve yielded similar ID_{50} values regardless of the route of administration [0.14×10^{-3} mg/kg (p.o.) and 0.13×10^{-3} mg/kg (i.p.)]. Compared with the p.o. ID_{50} of 40×10^{-3} mg/kg obtained for SR 48692 (Poncelet *et al.*, 1994a), this new derivative is a 100-fold more potent antagonist. These data led us to determine a duration of action of at least 6 hr after p.o. administration of SR 142948A (2×10^{-3} mg/kg). The potency of SR 142948A in antagonizing the NT-induced turning behavior underscores three important properties of this compound, in addition to its potent antagonism of NT effects, *i.e.*, oral bioavailability, long duration of action and crossing of the blood-brain barrier.

It has been clearly demonstrated that centrally injected NT, in addition to releasing DA, is able to release ACh in the striatum. Numerous data support the hypothesis that interactions between NT and DA, as well as between NT and ACh, may be increased under conditions disrupting the equilibrium between dopaminergic and cholinergic tone (*i.e.*, Parkinson disease or Huntington disease). In fact, the ACh release evoked by NT in the striatum is completely masked by the D_2 receptor-mediated inhibitory effect of the concomitant release of DA. To study this NT effect *in vivo*, intrastriatal injection of NT was carried out in rats pretreated with haloperidol (Steinberg *et al.*, 1995). As previously shown for SR 48692 (Steinberg *et al.*, 1995), i.p. injection of SR 142948A (0.1 mg/kg) before NT perfusion and haloperidol injection completely abolishes the ACh release elicited by the co-treatment with NT and haloperidol.

The modulating effect of NT on brain DA function has been well documented (Kasckow and Nemeroff, 1991). The NTR antagonist SR 48692 has recently been shown to increase or decrease the population response of rat A10 DA cells after acute or repeated administration, respectively, without affecting either their firing rate or bursting pattern (V. Santucci, submitted). In the same study, an increase in the A10 cell population response was also observed after direct injection of SR 48692 into the prefrontal cortex, suggesting that a potent cortical neurotensinergic influence is involved in the control of the level of excitability of A10 DA neurons. Compared with the effects of SR 48692 (Santucci *et al.*, submitted), the new antagonist is 10-fold more powerful in increasing the number of spontaneously active A10 DA cells. The mechanism of action of these two compounds could be similar, although the blockade of NTRs may also affect DA cells through other receptors or pathways (Jolas and Aghajanian, 1996). Further studies are needed to better delineate the electrophysiological effects of SR 142948A on DA function. In particular, it will be of interest to determine whether this compound, like SR 48692, is capable of selectively driving A10 DA cells into a state of depolarization block after repeated treatment (Santucci *et al*., submitted), which is currently considered to be a property of atypical antipsychotic drugs such as clozapine (Chiodo and Bunney, 1985). The induction of depolarization block of DA cells by antipsychotic drugs has been questioned because of the possible influence of anesthesia or of the cell-sampling method (Mereu *et al.*, 1995). However, acute blockade of neuropeptide receptors other than NTRs induces a decrease in the DA cell population response in unanesthetized animals through a mechanism different from that of depolarization block (Minabe *et al.*, 1996). Thus, the similarity of SR 142948A and SR 48692, at least in their acute effects on A10 cell population responses, suggests that the two compounds may share some of the properties of antipsychotic drugs.

Autoradiography coupled with electron-microscopy techniques has revealed, for [^{125}I-Tyr^3]NT injected in the rat striatum, receptor-dependent internalization followed by retrograde axonal transport to dopaminergic cell bodies (substantia nigra, ipsilateral to the striatum). This transport was found to be saturable and dependent on microtubular integrity (Castel *et al.*, 1994); it represents a long-distance signaling system in gene expression. It directly results in a signif-

icant increase in tyrosine hydroxylase mRNA in the substantia nigra (40%) (Burgevin *et al,* 1992). SR 142948A partially inhibits this retrograde axonal transport phenomenon, in a manner identical to that of SR 48692, concerning both the amplitude of the effect and the active dose-range (SR 48692 results reported by Steinberg *et al.*, 1994a).

Two other prominent central effects of NT in rodents, *i.e.*, hypothermia and analgesia (Bissette *et al*., 1976; Clineschmidt *et al.*, 1979), were not modified by SR 48692, suggesting that these effects might be mediated through a subtype of SR 48692-insensitive NTRs (Dubuc *et al*., 1994). Oral administration of SR 142948A partially inhibits the hypothermia elicited by i.c.v. injection of NT in mice and rats, with a bell-shaped dose-response relationship. A significant inhibitory effect appears in the same range of doses for both species (1–4 mg/kg), but at a higher dose (8 mg/kg) body temperature returns to that of the NT-treated group. The IC_{50} values of 1.7 mg/kg (rats) and 3.6 mg/kg (mice) were determined during the first phase in both species. In addition to the hypothermia, NT induces significant, opiate antagonist (*e.g.*, naloxone)-insensitive, antinociceptive activity in a variety of nociceptive tests (Coquerel *et al.*, 1988). The most sensitive test for NT was the writhing test, where the number of body stretches was counted in mice after i.p. injection of PBQ. These stretches were significantly reduced by i.c.v. injection of 2.5 ng of NT and returned to basal levels after treatment with increasing doses of SR 142948A (maximal efficacy at the 4 mg/kg dose). It is worth noting that the SR 142948A dose-effect relationships for hypothermia and analgesia were biphasic in similar dose ranges. In these two models of NT central effects, the potency of SR 142948A antagonism appears at doses that induce the reinstatement of rotations in the turning behavior model.

The different responses obtained with SR 48692 and SR 142948A strengthen the hypothesis of the existence of separate NTR subtypes implicated in the mediation of the antinociceptive and hypothermic effects of NT, as previously suggested by Poncelet *et al*. (1994a,b), Dubuc *et al*. (1994) and Labbé-Jullié *et al*. (1994). These actions do not occur in an isolated manner but involve a cascade of events that includes interaction with central neurotransmitter and neuropeptide systems. In our experiments, D_2 receptor blockade (spiroperidol at 0.1 mg/kg i.p.) completely abolishes the disappearance of effect seen for SR 142948A at doses above 4 mg/kg and highlights a probable implication of dopaminergic systems in these bell-shaped effects, as previously demonstrated in the turning behavior model. Nemeroff *et al*. (1980) suggested a possible interaction between NT and DA when describing the potentiation of the hypothermic effect of NT in rats by haloperidol or selective depletion of DA (6-hydroxydopamine lesions). On the other hand, Myers and Lee (1984) demonstrated that the effect of NT on body temperature regulation is independent of DA release. These conflicting suggestions cannot completely exclude an indirect interaction between neurotensinergic and dopaminergic systems. In spite of its efficiency in antagonizing these two NT effects, SR 142948A (like SR 48692) was unable to modify the DA release evoked in the nucleus accumbens after NT injection into the rat VTA, as measured by electrochemical detection coupled to polarography.

In summary, these data, taken together, allow us to conclude that SR 142948A can be considered as being representative of a second generation of potent, selective, nonpeptide antagonists of NTRs. It has oral bioavailability, crosses the blood-brain barrier and has long-lasting effects. Furthermore, unlike SR 48692, it inhibits the hypothermia and the antinociceptive effects of centrally injected NT, suggesting that SR 142948A may possibly interact with NTR subtypes. SR 142948A is more potent than SR 48692 in most of the *in vitro* and *in vivo* models, in particular in human receptor binding assays. The differences between these two compounds may help us to better understand the involvement of NT in pathophysiological regulatory mechanisms.

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