# Characterization of binding sites of a new neurotensin receptor antagonist, [$^3$H]SR 142948A, in the rat brain

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## Abstract

The present study describes the characterization of the binding properties and autoradiographic distribution of a new nonpeptide antagonist of neurotensin receptors, [$^3$H]SR 142948A (2-{5-(2,6-dimethoxyphenyl)-1-(4-(*N*-(3-dimethylaminopropyl)-*N*-methyl-carbamoyl)-2-isopropylphenyl)-1H-pyrazole-3-carbonyl]-amino}-adamantane-2-carboxylic acid, hydrochloride), in the rat brain. The binding of [$^3$H]SR 142948A in brain membrane homogenates was specific, time-dependent, reversible and saturable. [$^3$H]SR 142948A bound to an apparently homogeneous population of sites, with a $K_d$ of 3.5 nM and a $B_{max}$ value of 508 fmol/mg of protein, which was 80% higher than that observed in saturation experiments with [$^3$H]neurotensin. [$^3$H]SR 142948A binding was inhibited by SR 142948A, the related nonpeptide receptor antagonist, SR 48692 (2-{[1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]amino}-adamantane-2-carboxylic acid) and neurotensin. Saturation and competition studies in the presence or absence of the histamine H$_1$ receptor antagonist, levocabastine, revealed that [$^3$H]SR 142948A bound with similar affinities to both the levocabastine-insensitive neurotensin NT$_1$ receptors (20% of the total binding population) and the recently cloned levocabastine-sensitive neurotensin NT$_2$ receptors (80% of the receptors) ($K_d$ = 6.8 and 4.8 nM, respectively). The regional distribution of [$^3$H]SR 142948A binding in the rat brain closely matched the distribution of [$^{125}$I]neurotensin binding. In conclusion, these findings indicate that [$^3$H]SR 142948A is a new potent antagonist radioligand which recognizes with high affinity both neurotensin NT$_1$ and NT$_2$ receptors and represents thus an excellent tool to study neurotensin receptors in the rat brain. © 1998 Elsevier Science B.V.

*Keywords:* [$^3$H]SR 142948A; Neurotensin receptor; Nonpeptide receptor antagonist; Levocabastine; Receptor autoradiography; Brain

## 1. Introduction

Neurotensin is a 13-amino acid neuropeptide found in the central nervous system and peripheral tissues of numerous mammalian species (Emson et al., 1982; Mai et al., 1987). Neurotensin acts as a neurotransmitter–neuromodulator in a variety of physiological processes. In particular, neurotensin has been shown to play an important role in the modulation of midbrain dopamine transmission (Kasckow and Nemeroff, 1991); neurotensin is also involved in nociception, hypothermia and control of anterior pituitary hormone secretion (Rostène and Alexander, 1997). In the adult rat and mouse brain, neurotensin can bind to two different binding sites which can be distinguished by their affinity for neurotensin (Mazella et al.,

1983), as well as by their sensitivity to levocabastine, a histamine H$_1$ receptor antagonist (Schotte et al., 1986). In other species, including humans, rabbits and guinea pigs, only levocabastine-insensitive sites have been detected in the brain (Schotte et al., 1986). Until recently, it was believed that the physiological effects of neurotensin were mediated through a single class of G protein-coupled receptors, corresponding to the levocabastine-insensitive binding sites, cloned from rat brain (Tanaka et al., 1990) and human adenocarcinoma HT-29 cell line (Vita et al., 1993). In contrast, it was assumed that levocabastine-sensitive neurotensin binding sites lacked signalling activity and were thus considered as acceptor sites, devoid of function. Very recently, however, a novel neurotensin receptor (called NT$_2$) sensitive to levocabastine was cloned in the rat hypothalamus (Chalon et al., 1996) and mouse brain (Mazella et al., 1996). It also belongs to the family of G protein-coupled receptors and has about 40% homology

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with the previously cloned rat and human neurotensin receptors ($NT_1$). The biological function of the neurotensin $NT_2$ receptor remains to be determined.

A major advancement in the field of neurotensin research was provided by the discovery of the first highly potent and selective nonpeptide neurotensin receptor antagonist, SR 48692 (Gully et al., 1993). SR 48692 is orally active, crosses the blood brain barrier and has a long-lasting action; it shows higher affinity for neurotensin $NT_1$ than for $NT_2$ receptors. This antagonist can counteract the effects of neurotensin in numerous in vitro and in vivo assays (Gully et al., 1993); however, SR 48692 is unable to inhibit neurotensin-induced hypothermia and analgesia in rats and mice (Dubuc et al., 1994). This compound also fails to reverse dopamine release in the nucleus accumbens evoked by neurotensin injection in the ventral tegmental area (Steinberg et al., 1994), as well as the hypolocomotion induced by intracerebroventricular administration of the peptide (Pugsley et al., 1995). These findings suggest that these effects of neurotensin could be mediated through a neurotensin receptor subtype which is insensitive to SR 48692 (Le et al., 1996).

Although SR 48692 has proved an important pharmacological tool for studying neurotensin receptors and for exploring the existence of possible neurotensin receptor subtypes, it has certain properties that could limit its usefulness. In particular, SR 48692 has very low aqueous solubility and 100 times lower affinity for the rat brain when compared to the guinea pig brain (Gully et al., 1993). Thus, the tritiated ligand derived from this antagonist, [$^3$H]SR 48692, bound with high affinity to the guinea pig brain ($K_d = 2$ nM) (Betancur et al., 1995) and to cells transfected with the rat neurotensin $NT_1$ receptor ($K_d = 3$ nM) (Labbé-Jullié et al., 1995), but exhibited high levels of nonspecific binding when tested in adult rat brain homogenates or sections (unpublished observation).

Recently, Sanofi developed a second nonpeptide antagonist of neurotensin receptors, SR 142948A, which is chemically related to SR 48692 but has better solubility and increased affinity in the rat brain (Gully et al., 1997). SR 142948A recognizes with similar affinity (in the nanomolar range) both neurotensin $NT_1$ and $NT_2$ receptors (Gully et al., 1997). Interestingly, in contrast to SR 48692, SR 142948A blocked the hypothermia and analgesia induced by central injection of neurotensin, revealing a wider spectrum of action, probably through inhibition of different neurotensin receptor subtypes (Gully et al., 1997).

In the present study, we examined the binding properties of a newly developed tritiated form of SR 142948A ([$^3$H]SR 142948A) in adult rat brain membrane homogenates. Furthermore, we studied the autoradiographic distribution of [$^3$H]SR 142948A binding sites in the rat brain and compared it with that of [$^{125}$I]neurotensin binding. [$^3$H]SR 142948A binding was studied in the presence or absence of levocabastine, in order to determine its binding properties to neurotensin $NT_1$ and $NT_2$ receptors.

## 2. Materials and methods

### 2.1. Chemicals

SR 142948A (2-{[5-(2,6-dimethoxyphenyl)-1-(4-(N-(3-dimethylaminopropyl)-N-methylcarbamoyl)-2-isopropylphenyl)-1H-pyrazole-3-carbonyl]-amino}-adamantane-2-carboxylic acid, hydrochloride) (Fig. 1) and SR 48692 (2-{[1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl] amino}-adamantane-2-carboxylic acid) were synthesized at Sanofi Recherche (Montpellier, France). Both compounds were dissolved in dimethylsulfoxide and stored in aliquots at $-20$°C until the day of the experiment. [$^3$H]SR 142948A (83 Ci/mmol) was tritiated at Sanofi Recherche (Alnwick, Great Britain). [$^3$H]Neurotensin (104 Ci/mmol) was purchased from New England Nuclear (Les Ulis, France) and monoiodo-[$^{125}$I-Tyr$^3$]neurotensin (2000 Ci/mmol) was iodinated and purified as described previously (Sadoul et al., 1984). Unlabeled neurotensin was purchased from Neosystem Laboratories (Strasbourg, France). Levocabastine was kindly provided by Janssen Pharmaceutica (Beerse, Belgium) and was solubilized in ethanol.

### 2.2. Preparation of brain membrane homogenates

Male Sprague–Dawley rats (180–220 g, Charles River, Saint Aubain-les-Elboeuf, France) were killed by cervical dislocation. The whole brain (minus the cerebellum) was removed rapidly and homogenized in 10 volumes (original wet weight/volume) of 50 mM Tris–HCl ice-cold buffer (pH 7.4) for 30 s by using a polytron (setting 5). After 20 min of centrifugation at $50\,000 \times g$, the pellet was washed and again centrifuged as above. The final pellet was resuspended in binding assay buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% bovine serum albumin, 1 mM 1,10 orthophenanthroline (Sigma, Saint
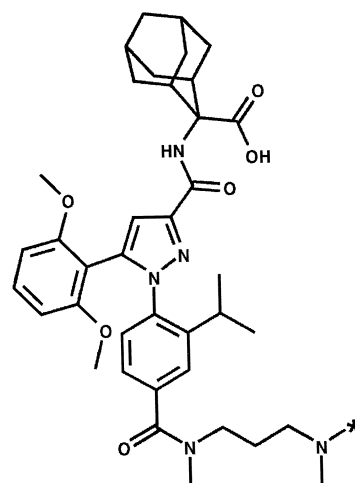


Fig. 1. Chemical structure of SR 142948A. *: location of the 3 tritium atoms.

Louis, MO), 5 mM dithiothreitol and 40 mg/l bacitracin and stored as aliquots in liquid nitrogen until used.

## 2.3. Binding assays in brain homogenates

Aliquots of brain membranes (300 $\mu$g/assay) were incubated in 0.5 ml (final volume) of binding assay buffer containing the appropriate concentrations of [$^3$H]SR 142948A and unlabeled drugs. After incubation at 20°C for 60 min, the assay medium was diluted with 4 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4) and filtered rapidly under reduced pressure through Whatman glass-fiber GF/B filters pretreated with 0.1% polyethylenimine. The filters were washed 3 times under the same conditions and transferred to vials containing 4 ml of scintillation cocktail. The bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 1 $\mu$M unlabeled SR 142948A.

Saturation experiments were carried out with increasing concentrations of [$^3$H]SR 142948A (0.05 to 16 nM). In association kinetic experiments, rat brain homogenates were incubated with [$^3$H]SR 142948A (2 nM) for various time periods. For dissociation studies, [$^3$H]SR 142948A was incubated for 30 min with brain membranes, unlabeled SR 142948A was then added at a final concentration of 1 $\mu$M and incubations were stopped at the indicated times. Competition studies were conducted with a single concentration of [$^3$H]SR 142948A (2 nM) and at least 10 concentrations of unlabeled ligands. In addition, saturation and competition studies with [$^3$H]SR 142948A were performed in the presence of a constant concentration of levocabastine (10 $\mu$M), which selectively inhibits binding to neurotensin NT$_2$ receptors. The effect of guanyl nucleotides on [$^3$H]SR 142948A binding was examined by adding increasing concentrations of the nonhydrolyzable GTP analog 5'-guanylylimidodiphosphate (Gpp(NH)p) to the binding assay. [$^3$H]neurotensin binding to rat brain membranes was performed as described previously (Goedert et al., 1984), in the presence or absence of levocabastine (10 $\mu$M). Nonspecific binding was determined by incubation with 1 $\mu$M neurotensin. All experiments were performed 2 or 3 times in triplicate. Data from association, saturation and competition studies were analyzed by using a nonlinear regression program, LIGAND (Munson and Rodbard, 1980). $K_i$ values were calculated according to the Cheng and Prusoff (1973) equation.

## 2.4. Autoradiography of [$^3$H]SR 142948A binding sites

Adult male rats were killed by decapitation, the brain was removed rapidly, frozen on dry ice and stored at −80°C. Coronal sections (20 $\mu$m thick) were cut on a cryostat at −16°C, mounted on slides (Superfrost plus, Menzel-Glaser, Madison, WI) and stored at −20°C until used. [$^3$H]SR 142948A binding was performed by incubating the sections for 60 min at room temperature with 300 $\mu$l of 2 nM [$^3$H]SR 142948A in 50 mM Tris–HCl buffer

(pH 7.4), containing 1 mM EDTA, 0.1% bovine serum albumin, 40 mg/ml bacitracin and 0.5 mM 1,10 or-thophenanthroline, in the presence or absence of 1 $\mu$M levocabastine. Additional sections were incubated with 10 $\mu$M SR 142948A for the determination of nonspecific binding. After incubation, the sections were washed 3 times for 10 min each at 4°C in 50 mM Tris–HCl buffer (pH 7.4), containing 1 mM EDTA and 0.1% bovine serum albumin. Slides were then dipped briefly in distilled water and dried under a stream of air. Sections were placed in X-ray cassettes, apposed to Hyperfilm-$^3$H (Amersham, France) for 3 weeks and developed by standard photographic procedures.

## 2.5. Autoradiography of [$^{125}$I]neurotensin binding sites

[$^{125}$I]Neurotensin binding was performed as described previously (Moyse et al., 1987). Briefly, slide-mounted brain sections were incubated for 60 min at 4°C with 300 $\mu$l of 0.3 nM [$^{125}$I]neurotensin in 50 mM Tris–HCl buffer (pH 7.4), containing 5 mM MgCl$_2$, 0.2% bovine serum albumin and 0.5 mM 1,10 orthophenanthroline, in the presence or absence of 1 $\mu$M levocabastine. Nonspecific binding was determined in the presence of 10 $\mu$M unlabeled neurotensin. The sections were washed 4 times for 2 min each at 4°C in 40 mM Tris–HCl buffer (pH 7.4), dipped briefly in distilled water and dried. Radiolabeled sections were exposed to Hyperfilm-$\beta$max (Amersham) for 1 week.

## 2.6. Densitometric analysis

Quantitative optical density measurements of film autoradiographs were carried out with a computer-based image analysis system (HISTO-RAG, Biocom, Les Ulis, France). Optical densities of nonspecific binding were subtracted from total binding to obtain specific binding. Measurements were performed bilaterally on 4 brain sections per level. Values were expressed as nCi/mg tissue by using autoradiographic $^3$H and $^{125}$I micro-scales (Amersham) that were exposed together with the tissue sections. Brain structures were identified according to the atlas of Paxinos and Watson (1986).

## 3. Results

### 3.1. Biochemical profile of [$^3$H]SR 142948A binding to rat brain membrane homogenates

#### 3.1.1. Tissue concentration linearity

Fig. 2 shows the total, nonspecific and specific binding of [$^3$H]SR 142948A to rat brain homogenates as a function of membrane protein concentration. The specific binding of [$^3$H]SR 142948A measured at 2 nM was linear with increasing protein concentration until 400 $\mu$g of protein/tube.
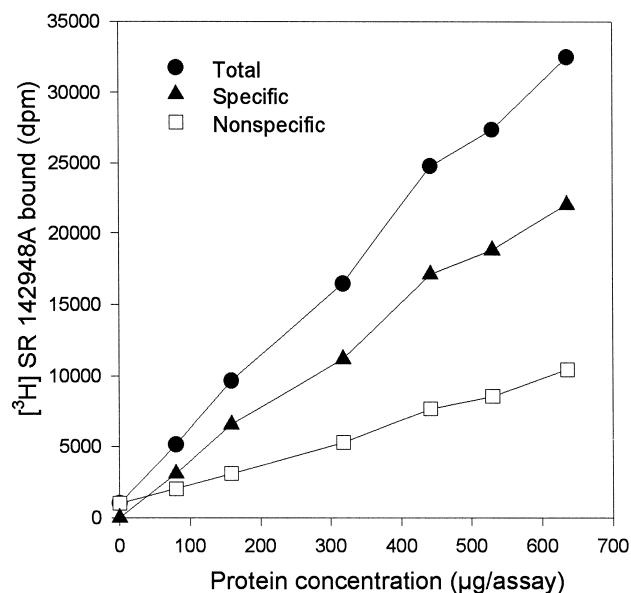
Fig. 2. [³H]SR 142948A binding as a function of increasing protein concentrations. Various concentrations of rat brain membranes were incubated with 2 nM [³H]SR 142948A for 60 min at 20°C. Nonspecific binding was determined with 1 $\mu$M unlabeled SR 142948A. Specific binding was defined as the difference between total and nonspecific binding. The data represent the mean of triplicate determinations.

### 3.1.2. Saturation studies

Fig. 3 shows a saturation isotherm of the binding of [³H]SR 142948A to rat brain membranes. Analysis of the saturation curves by computer-assisted nonlinear regression or by Scatchard analysis (Fig. 3, inset) revealed a single class of high-affinity binding sites ($K_d = 3.48 \pm 0.22$
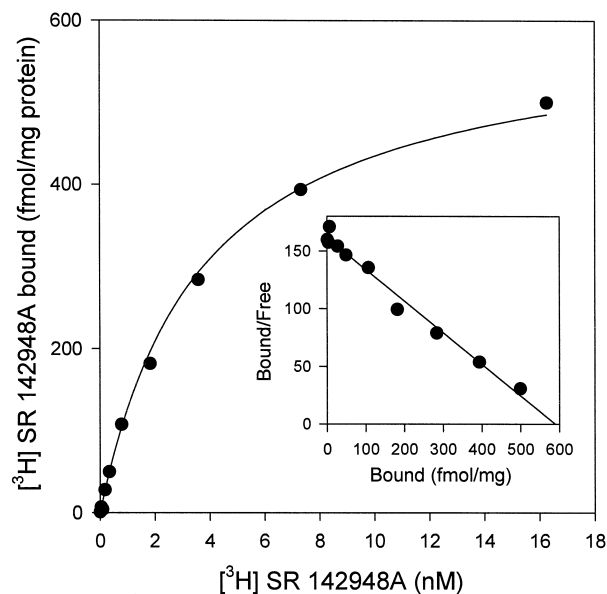
nM; mean $\pm$ S.E.M., $n = 3$) for radioligand concentrations ranging from 0.05 to 16 nM, with a maximal binding capacity ($B_{max}$) of 508.4 $\pm$ 45.0 fmol/mg of protein.

### 3.1.3. Kinetic studies

The specific binding of [³H]SR 142948A to rat brain membranes was time dependent, reaching a steady state in about 60–80 min at 20°C (Fig. 4A). Fig. 4B illustrates the rate of dissociation, measured at various time intervals after addition of 1 $\mu$M unlabeled SR 142948A at equilibrium binding. The dissociation kinetics revealed a first-order process with a dissociation rate constant ($k_{-1}$) of 0.021 min$^{-1}$ (mean, $n = 2$). The observed association rate constant ($k_{obs}$) was 0.033 min$^{-1}$ and the kinetic association constant ($k_1$), calculated from the equation $k_1 = (k_{obs}$







Fig. 3. Saturation analysis and Scatchard plot (inset) of [³H]SR 142948A binding to rat brain membranes. Membranes were incubated for 60 min at 20°C over a concentration range of [³H]SR 142948A (0.05 to 16 nM); nonspecific binding was defined with 1 $\mu$M unlabeled SR 142948A. The data shown are from one of 3 experiments performed in triplicate.
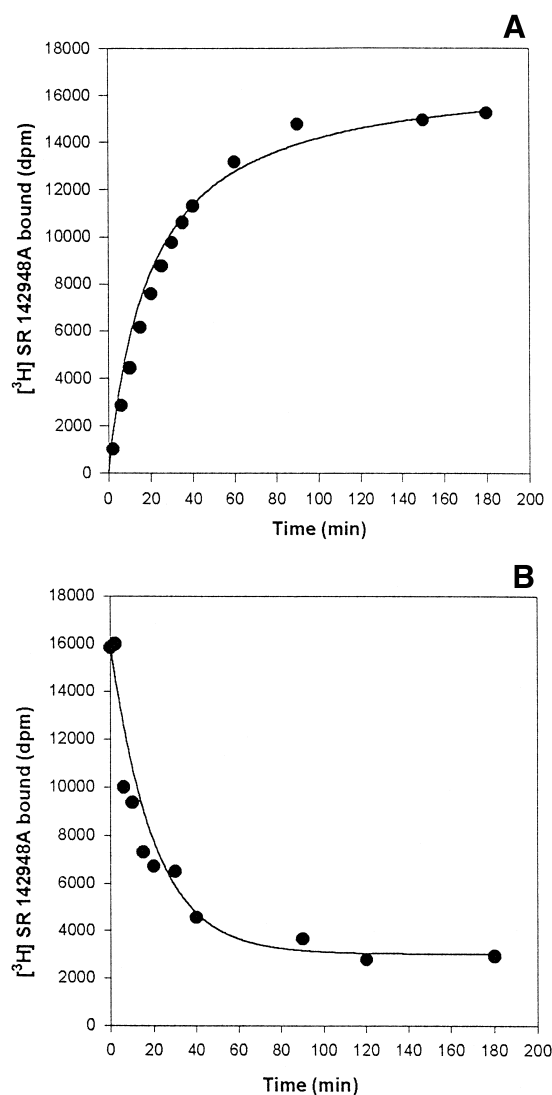
Fig. 4. Association (A) and dissociation (B) kinetics of specific [³H]SR 142948A binding to rat brain membranes. (A) The association of 2 nM [³H]SR 142948A binding was determined at various time intervals. (B) Time course of dissociation of [³H]SR 142948A binding, initiated with 1 $\mu$M unlabeled SR 142948A. The points shown are means of triplicate determinations from a representative experiment.
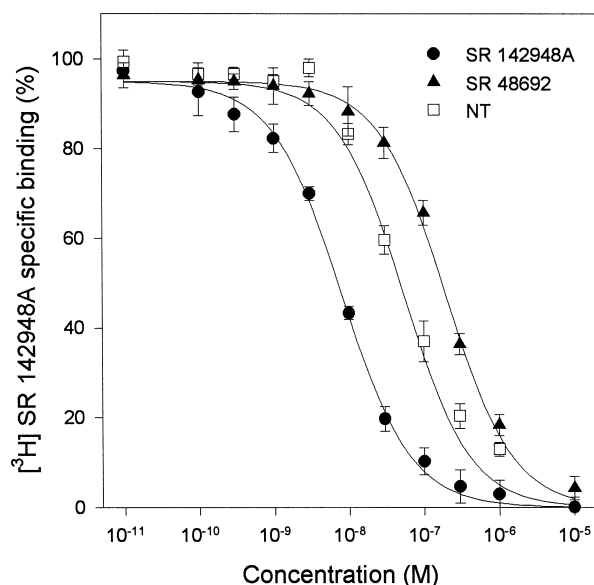
Fig. 5. Inhibition of specific [³H]SR 142948A binding to rat brain membranes by increasing concentrations of SR 142948A, SR 48692 and neurotensin. Results represent the means ± S.E.M. of 3 independent experiments performed in triplicate.

$-k_1)/([^3H]SR\ 142948A)$, was $0.006 \times 10^9\ M^{-1}\ min^{-1}$. The dissociation constant ($K_d$) calculated from the ratio $k_{-1}/k_1$ was 3.5 nM, similar to the dissociation constant determined in saturation studies.
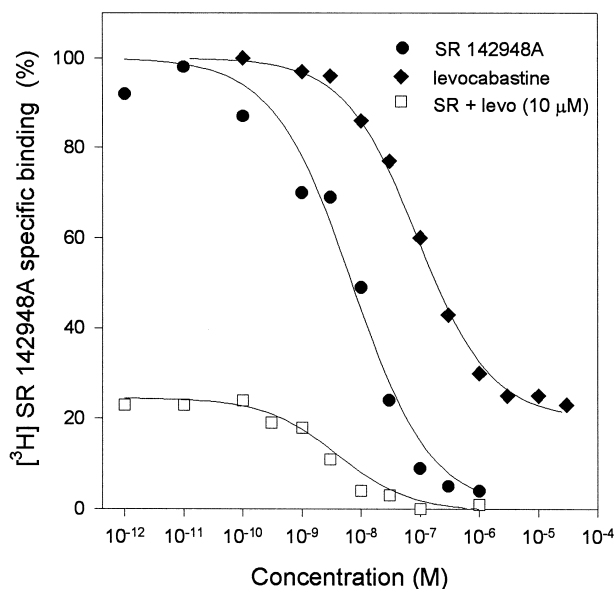


Fig. 6. Inhibition of specific [³H]SR 142948A binding to rat brain membranes by SR 142948A, levocabastine and SR 142948A in the presence of a constant concentration of levocabastine (10 μM). Specific binding of [³H]SR 142948A without levocabastine represents 100%. In the absence of levocabastine, SR 142948A (●) inhibited [³H]SR 142948A binding to neurotensin NT₁ and NT₂ receptors. Levocabastine (♦) inhibited binding of [³H]SR 142948A to NT₂ receptors (80% of the receptors). In the presence of 10 μM levocabastine, SR 142948A (□) inhibited [³H]SR 142948A binding to NT₁ receptors (20% of the receptors). The data shown are from a single experiment performed in triplicate. The experiment was repeated 3 times with similar results.
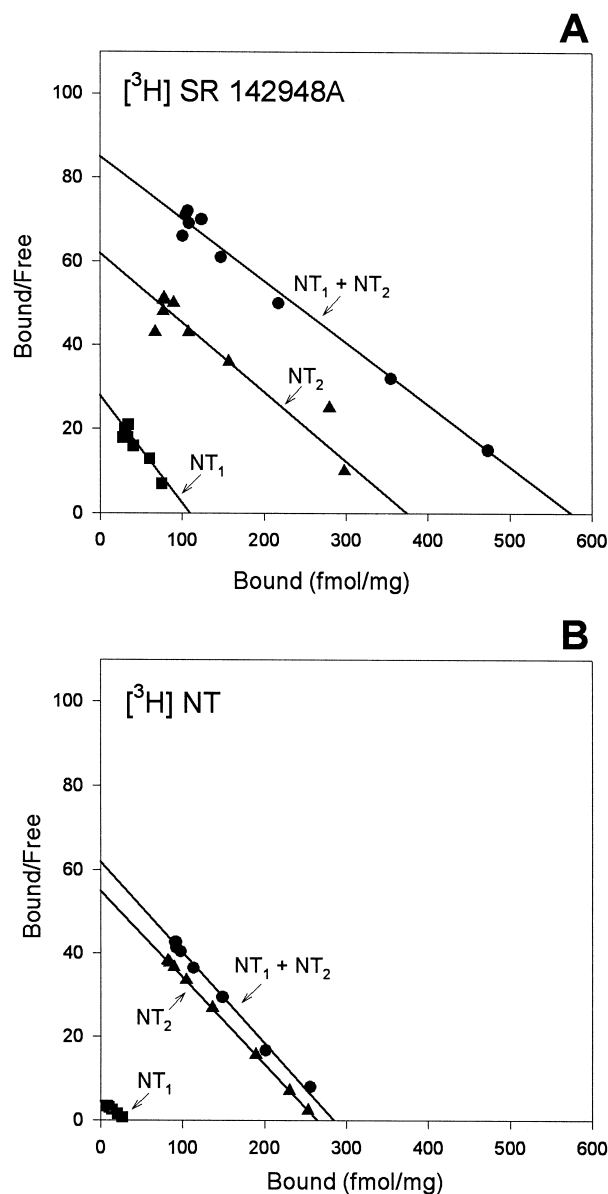


Fig. 7. Scatchard analysis of saturation of [³H]SR 142948A (A) and [³H]neurotensin (B) binding to rat brain membranes performed with or without 10 μM levocabastine. [³H]SR 142948A and [³H]neurotensin bound to neurotensin NT₁ and NT₂ receptors; the addition of 10 μM levocabastine to the binding assay blocked levocabastine-sensitive NT₂ receptors and revealed NT₁ receptors. The NT₂ receptor data were calculated as the difference between the results obtained in the absence and in the presence of levocabastine. The values are from typical experiments and represent the means of triplicate determinations.

### 3.1.4. Competition studies

Fig. 5 shows the inhibition of [³H]SR 142948A binding by increasing concentrations of unlabeled SR 142948A, SR 48692 and neurotensin in rat brain homogenates. The $K_i$ value obtained for unlabeled SR 142948A, $5.0 \pm 0.4$ nM, was close to the values determined in kinetic studies (Hill coefficient, $n_H = 0.98 \pm 0.01$). The binding of [³H]SR 142948A to rat brain membranes was fully displaced by the natural ligand neurotensin, as well as by the previously described neurotensin receptor antagonist SR 48692, a

nonpeptide molecule chemically related to SR 142948A. These competition curves gave $K_i$ values of $32.8 \pm 5.9$ nM ($n_H = 0.99 \pm 0.10$) for neurotensin and $123.6 \pm 15.7$ nM ($n_H = 0.95 \pm 0.06$) for SR 48692.

### 3.1.5. Effect of levocabastine on [³H]SR 142948A and [³H]neurotensin binding

The addition of increasing concentrations of levocabastine to the [³H]SR 142948A binding assay resulted in progressive blockade of neurotensin $NT_2$ receptors (Fig. 6). Concentrations greater than 1 $\mu$M levocabastine completely inhibited the binding of [³H]SR 142948A to $NT_2$ receptors, which constituted 80% of the whole population of sites. The presence of 10 $\mu$M levocabastine in the [³H]SR 142948A binding assay revealed neurotensin $NT_1$ receptors, which were recognized by SR 142948A with an

$IC_{50}$ value of $4.0 \pm 0.3$ nM, in the same nanomolar range as for experiments without levocabastine ($IC_{50} = 7.0 \pm 1.5$ nM).

Saturation experiments were also performed in the presence or absence of 10 $\mu$M levocabastine with a fixed concentration of [³H]SR 142948A and increasing concentrations of unlabeled SR 142948A. Scatchard analysis of [³H]SR 142948A binding data in the absence of levocabastine yielded a linear plot (Fig. 7A), indicating the presence of a homogeneous population of binding sites ($K_d = 6.1 \pm 1.6$ nM, $B_{max} = 527 \pm 22$ fmol/mg protein; $n = 3$). However, the parallel leftward shift of this straight line obtained in the presence of 10 $\mu$M levocabastine indicated binding to neurotensin $NT_1$ receptors ($K_d = 3.4 \pm 0.5$ nM, $B_{max} = 104 \pm 8$ fmol/mg protein) and, by difference, revealed neurotensin $NT_2$ receptors ($K_d = 8.5 \pm 4.1$ nM,
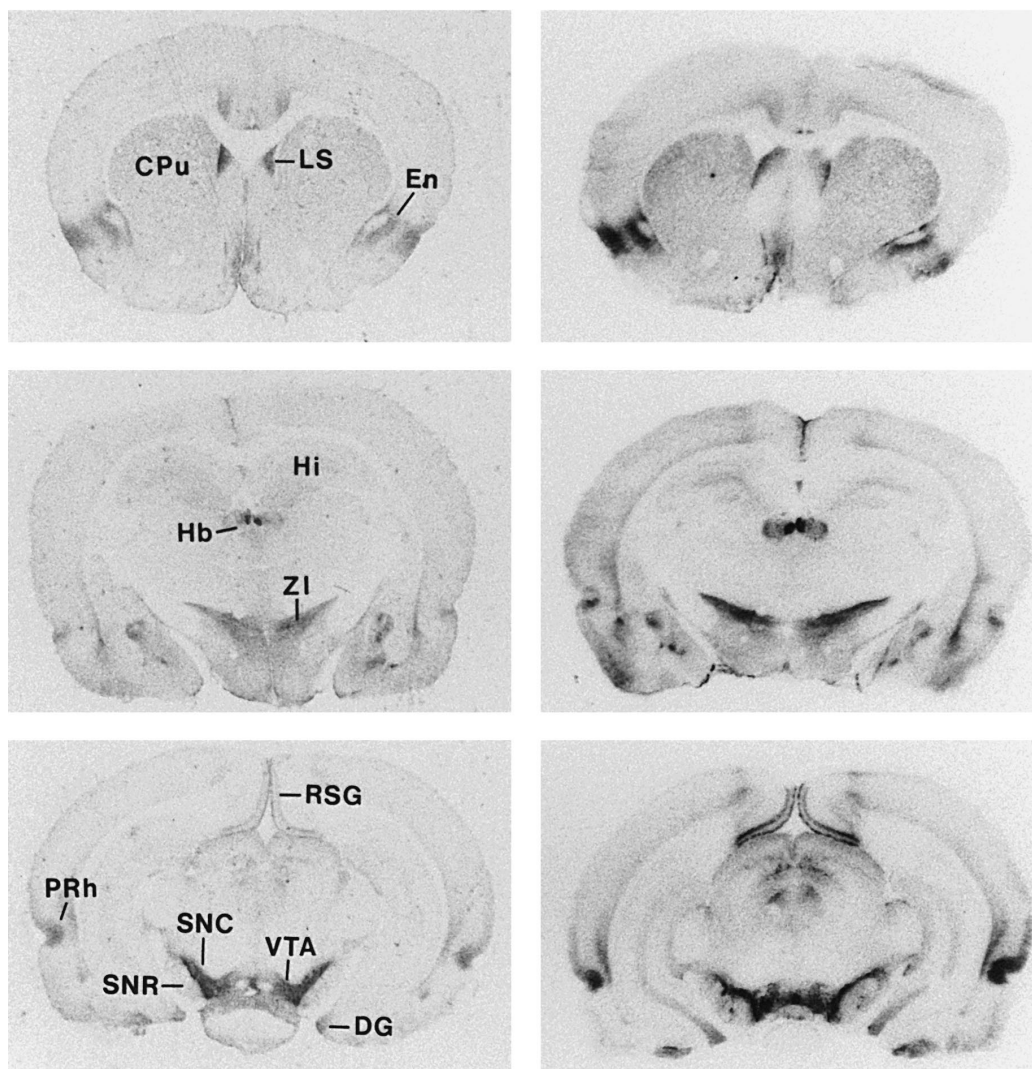


Fig. 8. Autoradiographic distribution of [³H]SR 142948A (left) and [¹²⁵I]neurotensin (right) binding sites in coronal sections of the rat brain. Photographs show [³H]SR 142948A and [¹²⁵I]neurotensin binding in the absence of levocabastine. Nonspecific binding was determined in the presence of 10 $\mu$M unlabeled SR 142948A or neurotensin, respectively, and was indistinguishable from background. Abbreviations: CPu = caudate putamen; DG = ventral dentate gyrus; En = endopiriform nucleus; Hb = habenula; Hi = hippocampus; LS = lateral septum; Prh = perirhinal area; RSG = retrosplenial granular cortex; SNC = substantia nigra, pars compacta; SNR = substantia nigra, pars reticulata; VTA = ventral tegmental area; ZI = zona incerta.

$B_{max} = 422 \pm 58$ fmol/mg protein). These data confirmed that [³H]SR 142948A exhibits similar affinities for the two subtypes of neurotensin receptors. Similar experiments performed with [³H]neurotensin (Fig. 7B) revealed $K_d$ values comparable to those of [³H] SR 142948A (6.8 and 4.8 nM for $NT_1$ and $NT_2$ receptors, respectively). However, [³H]neurotensin bound to a lower number of binding sites than [³H]SR 142948A ($B_{max}$ values of 32 and 265 fmol/mg for $NT_1$ and $NT_2$ receptors, respectively).

### 3.1.6. Effect of guanyl nucleotides on [³H]SR 142948A binding

In order to determine whether the binding of [³H]SR 142948A was sensitive to guanyl nucleotides, we examined the affinity of the radioligand in the presence of Gpp(NH)p, a nonhydrolyzable analog of GTP. The addition of Gpp(NH)p in concentrations up to 100 $\mu$M did not modify the specific binding of [³H]SR 142948A to rat brain membranes (data not shown).

### 3.2. Autoradiographic localization of [³H]SR 142948A binding sites in the rat brain: Comparison with [¹²⁵I]neurotensin binding

Preliminary experiments showed that the characteristics of [³H]SR 142948A binding to rat midbrain sections were similar to those observed for brain membrane homogenates. [³H]SR 142948A binding was saturable and reached a steady state by 60 min. Specific [³H]SR 142948A binding was approximately 90% of the total binding, as

Table 1
Regional distribution of [³H]SR 142948A and [¹²⁵I]neurotensin binding sites in the rat brain, in the presence or absence of levocabastine

| Brain area | Specific binding | | | |
|---|---|---|---|---|
| | [³H]SR 142948A | | [¹²⁵I]neurotensin | |
| | − levo | + levo | − levo | + levo |
| **Cerebral cortex** | | | | |
| Frontal cortex | $1.91 \pm 0.11$ | $1.12 \pm 0.02$ | $0.39 \pm 0.03$ | $0.29 \pm 0.01$ |
| Anterior cingulate cortex | $6.61 \pm 0.27$ | $6.41 \pm 0.14$ | $2.01 \pm 0.16$ | $1.79 \pm 0.14$ |
| Dorsal peduncular cortex | $9.44 \pm 0.18$ | $7.67 \pm 0.27$ | $2.47 \pm 0.39$ | $1.80 \pm 0.09$ |
| Agranular insular cortex | $7.58 \pm 0.21$ | $5.66 \pm 0.51$ | $1.62 \pm 0.19$ | $1.05 \pm 0.09$ |
| Dorsal endopiriform nucleus | $5.06 \pm 0.12$ | $4.59 \pm 0.09$ | $1.82 \pm 0.13$ | $1.15 \pm 0.07$ |
| Parietal cortex | $1.84 \pm 0.05$ | $0.82 \pm 0.05$ | $0.40 \pm 0.02$ | $0.27 \pm 0.01$ |
| Perirhinal area | $10.54 \pm 0.29$ | $8.98 \pm 0.22$ | $2.51 \pm 0.32$ | $2.29 \pm 0.05$ |
| Retrosplenial granular cortex | $3.11 \pm 0.09$ | $1.98 \pm 0.12$ | $1.07 \pm 0.03$ | $0.84 \pm 0.06$ |
| Temporal cortex | $2.22 \pm 0.05$ | $1.01 \pm 0.06$ | $0.47 \pm 0.01$ | $0.33 \pm 0.01$ |
| **Forebrain** | | | | |
| Septohippocampal nucleus | $5.90 \pm 0.27$ | $5.50 \pm 0.29$ | $2.09 \pm 0.47$ | $2.01 \pm 0.17$ |
| Lateral septal nucleus, dorsal part | $5.99 \pm 0.25$ | $4.65 \pm 0.10$ | $1.13 \pm 0.02$ | $1.02 \pm 0.05$ |
| **Basal ganglia** | | | | |
| Caudate putamen (striatum) | $2.57 \pm 0.13$ | $2.12 \pm 0.06$ | $0.58 \pm 0.02$ | $0.51 \pm 0.02$ |
| Accumbens nucleus, shell | $2.62 \pm 0.08$ | $1.66 \pm 0.07$ | $0.52 \pm 0.02$ | $0.35 \pm 0.01$ |
| Accumbens nucleus, core | $2.39 \pm 0.09$ | $1.55 \pm 0.06$ | $0.55 \pm 0.03$ | $0.37 \pm 0.01$ |
| **Amygdala** | | | | |
| Central amygdaloid nucleus | $5.00 \pm 0.33$ | $3.10 \pm 0.09$ | $1.29 \pm 0.09$ | $0.93 \pm 0.02$ |
| Posteromedial cortical amygdaloid nucleus | $9.17 \pm 0.38$ | $8.06 \pm 0.26$ | $1.83 \pm 0.20$ | $1.60 \pm 0.12$ |
| **Diencephalon** | | | | |
| Zona incerta | $7.84 \pm 0.14$ | $5.67 \pm 0.46$ | $1.61 \pm 0.06$ | $1.32 \pm 0.03$ |
| Lateral habenular nucleus | $5.41 \pm 0.20$ | $4.01 \pm 0.11$ | $1.20 \pm 0.06$ | $0.80 \pm 0.04$ |
| Medial habenular nucleus | $10.68 \pm 0.48$ | $9.01 \pm 0.50$ | $2.63 \pm 0.14$ | $1.92 \pm 0.07$ |
| **Hippocampal formation** | | | | |
| Dentate gyrus, ventral part | $8.41 \pm 0.44$ | $7.64 \pm 0.31$ | $1.19 \pm 0.08$ | $0.98 \pm 0.14$ |
| **Midbrain** | | | | |
| Substantia nigra, pars compacta | $14.25 \pm 1.08$ | $11.34 \pm 1.08$ | $2.38 \pm 0.10$ | $2.05 \pm 0.26$ |
| Substantia nigra, pars reticulata | $3.21 \pm 0.13$ | $2.79 \pm 0.14$ | $0.99 \pm 0.05$ | $0.83 \pm 0.07$ |
| Ventral tegmental area | $15.10 \pm 1.08$ | $9.77 \pm 0.97$ | $2.18 \pm 0.17$ | $1.71 \pm 0.19$ |
| Superficial gray layer of the superior colliculus | $4.10 \pm 0.13$ | $2.28 \pm 0.08$ | $0.82 \pm 0.03$ | $0.64 \pm 0.03$ |

[³H]SR 142948A and [¹²⁵I]neurotensin specific binding to neurotensin $NT_1$ and $NT_2$ receptors was determined on rat brain sections by autoradiography, in the presence (+ levo) or absence (− levo) of levocabastine (1 $\mu$M). The occlusion of neurotensin $NT_2$ receptors by levocabastine allowed detection of $NT_1$ receptors. The difference between binding in the absence and in the presence of levocabastine represents binding to $NT_2$ receptors. Optical densities were measured bilaterally on 4 brain sections per level and expressed as mean $\pm$ S.E.M. nCi/mg tissue.

determined with 2 nM radioligand. Analysis of competition studies performed on rat midbrain sections showed that SR 142948A, SR 48692 and neurotensin induced a dose-dependent and complete inhibition of [$^3$H]SR 142948A binding (data not shown).

Fig. 8 shows the regional distribution of [$^3$H]SR 142948A and [$^{125}$I]neurotensin binding sites in the rat brain. The highest density of [$^3$H]SR 142948A and [$^{125}$I]neurotensin binding was present in the midbrain, in the ventral tegmental area and substantia nigra pars compacta. Intense labeling was also observed in the perirhinal area as well as in the dorsal peduncular, anterior cingulate and agranular insular cortices, posteromedial cortical amygdaloid nucleus, medial habenula and ventral dentate gyrus. The endopiriform nucleus, septohippocampal nucleus, central amygdaloid nucleus and zona incerta exhibited moderate levels of binding. Finally, a low density of binding sites was observed in the frontal, parietal, temporal and retrosplenial granular cortices, caudate putamen, nucleus accumbens, lateral septum, hypothalamus, hippocampus, substantia nigra pars reticulata and in the superficial gray layer of the superior colliculus.

Table 1 shows the comparative distribution of [$^3$H]SR 142948A and [$^{125}$I]neurotensin binding sites in the rat brain, in the presence or absence of levocabastine. An excellent correlation between the regional distribution of the two ligands was observed, in agreement with the autoradiographic localization of neurotensin receptors in rat brain described previously (Moyse et al., 1987). The presence of levocabastine in the incubation buffer inhibited binding to neurotensin $NT_2$ receptors and decreased the amount of labeling with [$^3$H]SR 142948A and [$^{125}$I]neurotensin in all brain regions studied, indicating that both ligands labeled neurotensin $NT_1$ and $NT_2$ receptors. This result is in agreement with the ubiquitous distribution of $NT_2$ receptors in the rat brain (Schotte et al., 1986).

## 4. Discussion

The binding of the nonpeptide neurotensin receptor antagonist [$^3$H]SR 142948A to rat brain membranes was rapid, tissue concentration and time dependent, saturable and reversible. Scatchard analyses of saturation experiments indicated that [$^3$H]SR 142948A binds with high affinity ($K_d = 3.5$ nM) and apparently recognizes a single class of binding sites. The number of sites labeled by [$^3$H]SR 142948A ($B_{max} = 508$ fmol/mg protein) was 80% greater than the $B_{max}$ value determined with [$^3$H]neurotensin under the same experimental conditions (297 fmol/mg protein). Competition experiments with unlabeled SR 142948A yielded a Hill coefficient near unity, further suggesting that the antagonist bound to an apparently homogeneous population of binding sites.

The potencies of the neurotensin receptor antagonists SR 142948A and SR 48692 in inhibiting specific [$^3$H]SR

142948A binding ($K_i = 5$ and 123.6 nM, respectively) were similar to their previously reported potencies in displacing [$^{125}$I]neurotensin binding in adult rat brain homogenates ($IC_{50} = 3.96$ and 82 nM for SR 142948A and SR 48692, respectively) (Gully et al., 1997). In contrast, the potency of the natural peptide agonist neurotensin for inhibiting [$^3$H]SR 142948A binding ($K_i = 32.8$ nM) was 10-fold lower than its potency in displacing [$^{125}$I]neurotensin binding ($IC_{50} = 3.2$ nM). This is consistent with results obtained with other receptor systems, demonstrating that estimates of agonist affinity are lower when an antagonist rather than an agonist radioligand is displaced. Indeed, a reduced potency for agonists to compete against radiolabeled antagonist ligands has been observed previously for neurotensin receptors in the guinea pig brain (Betancur et al., 1995), as well as for muscarinic cholinergic receptors (Waelbroeck et al., 1982), cholecystokinin $CCK_A$ (Chang et al., 1986; Talkad et al., 1994) and $CCK_B$ receptors (Chang et al., 1989), tachykinin $NK_1$ receptors (McLean et al., 1991) and 5-$HT_2$ receptors (Teitler et al., 1990). These findings have been interpreted as indicating the existence of different conformational states of the same receptor, with different affinities for agonist and antagonist ligands (Schwartz et al., 1995).

The higher number of receptors detected with [$^3$H]SR 142948A when compared with the number detected with the agonist radioligand, [$^3$H]neurotensin, also supports this hypothesis. The ability of radiolabeled nonpeptide antagonists to recognize a larger number of receptors, characterized by low affinity for the agonist, than agonist-derived radioligands appears to be a common phenomenon. For instance, we showed previously that in the guinea pig brain the number of binding sites labeled by the antagonist [$^3$H]SR 48692 exceeded by 20-fold the number of receptors labeled with the agonist [$^{125}$I]neurotensin (Betancur et al., 1995). The binding sites detected by [$^3$H]SR 48692 were characterized by a low affinity for neurotensin and were insensitive to GTP, suggesting that they represent the uncoupled form of the neurotensin receptor. These data, together with current hypotheses on the molecular interactions of ligands with their receptors, suggest that agonists bind with high affinity to the active receptor conformation, whereas antagonists bind with higher affinity to the inactive conformation (Schwartz et al., 1995). Thus, peptide agonists and nonpeptide antagonists act as allosteric competitive ligands by binding in a mutually exclusive fashion to sites occurring in different receptor conformations. In support of this model, recent site-directed mutational studies of the rat neurotensin $NT_1$ receptor showed that mutations in the N-terminal part eliminate neurotensin binding without affecting the binding of [$^3$H]SR 48692 (Labbé-Jullié et al., 1995). This finding suggests the existence of distinct agonist and antagonist binding domains on the neurotensin $NT_1$ receptor, similarly to what has been reported for several other neuropeptide receptors (for review, see Betancur et al., 1997).

Levocabastine is a nonpeptide antagonist of histamine $H_1$ receptors that is structurally unrelated to neurotensin (Stockbroekx et al., 1986), but binds also to neurotensin $NT_2$ receptors (Schotte et al., 1986). The recent cloning of the mouse $NT_2$ receptor and its expression in *Xenopus* oocytes indicated that levocabastine acts as an agonist in this system, since it triggers a $Cl^-$ inward current, as does neurotensin (Mazella et al., 1996). Saturation and competition experiments performed with or without levocabastine indicated that $[^3H]SR$ 142948A bound with high affinity to neurotensin $NT_1$ and $NT_2$ receptors ($K_d$ = 3.4 and 8.5 nM, respectively). Indeed, the addition of levocabastine to the $[^3H]SR$ 142948A binding assay caused a parallel leftward shift of the Scatchard plot, indicating the displacement of $[^3H]SR$ 142948A binding from levocabastine-sensitive $NT_2$ receptors. The similar affinity of $[^3H]SR$ 142948A for both subtypes of neurotensin receptors explains the apparently homogeneous population of binding sites detected with this ligand in the absence of levocabastine. Moreover, our results indicate that neurotensin $NT_2$ receptors constitute 80% of the whole population of sites labeled by $[^3H]SR$ 142948A ($B_{max}$ = 104 and 422 fmol/mg protein for $NT_1$ and $NT_2$ receptors, respectively) on rat brain membranes.

Guanyl nucleotides differentially affect agonist and antagonist binding in several neurotransmitter receptor systems. Accordingly, guanyl nucleotides have been reported to significantly reduce $[^{125}I]$neurotensin binding to $NT_1$ receptors, by interfering with the formation of the high-affinity agonist-receptor-G protein ternary complex (Hermans et al., 1996). In contrast, the present study showed that addition of Gpp(NH)p had no effect on specific $[^3H]SR$ 142948A binding. This finding is consistent with previous data showing that antagonist ligands bind with high affinity to the G protein-uncoupled state of receptors (Teitler et al., 1990; Rosenkilde et al., 1994; Betancur et al., 1995). It should be noted, however, that the levocabastine-sensitive neurotensin binding site is insensitive to GTP (Vincent, 1995), although recent data indicate that the cloned mouse neurotensin $NT_2$ receptor is coupled functionally to phospholipase C when expressed in oocytes (Mazella et al., 1996). Other studies have shown that an absence of GTP-sensitive binding does not necessarily indicate failure to activate a second messenger cascade (Chung et al., 1988; Maeda et al., 1990; Hermans et al., 1996).

The different sensitivity to Gpp(NH)p of neurotensin $NT_1$ and $NT_2$ receptors is particularly interesting in view of the fact that the lowest homology between the two neurotensin receptors is in their third cytoplasmic loop and C-terminal domain (Chalon et al., 1996; Mazella et al., 1996), two regions implicated in the coupling to G proteins (Yamada et al., 1994; Hermans et al., 1996). Furthermore, it has been proposed that the extremely high number of Ser/Thr residues in the third intracytoplasmic loop and the C-terminal domain of the neurotensin $NT_2$ receptor protein could be associated with a basal phosphorylated state resulting in desensitization of the receptor (Mazella et al., 1996). This could result in a higher proportion of receptors in the uncoupled form expressed in the membrane and might explain the insensitivity of the $NT_2$ receptor to GTP analogs. The use of recently developed radiolabeled agonist ligands specific for the G protein-coupled state of neurotensin receptors (Gaudriault et al., 1996), in the presence or absence of levocabastine, could help to determine the different states of coupling of neurotensin $NT_1$ and $NT_2$ receptors.

The autoradiographic distribution of $[^3H]SR$ 142948A binding in sections of rat brain was consistent with its selective binding to neurotensin receptors. The heterogeneous pattern of specific $[^3H]SR$ 142948A binding closely matched the localization of $[^{125}I]$neurotensin binding observed in adjacent sections. The addition of levocabastine to the incubation buffer resulted in a small and diffuse reduction of $[^3H]SR$ 142948A and $[^{125}I]$neurotensin binding in all brain structures studied, in agreement with previous studies indicating that neurotensin $NT_2$ receptors are distributed throughout the rat central nervous system (Schotte et al., 1986; Kitabgi et al., 1987) and are predominantly associated with glial cells (Schotte et al., 1988). The ubiquitous distribution of neurotensin $NT_2$ receptors contrasts with the highly regional localization of $NT_1$ receptors in the brain. Indeed, $NT_1$ receptors are particularly abundant in brain regions rich in dopamine neurons, such as the substantia nigra and the ventral tegmental area, as well as in certain cortical areas. These results are in agreement with the previously described distribution of $[^{125}I]$neurotensin binding (Moyse et al., 1987) and neurotensin $NT_1$ receptor mRNA (Nicot et al., 1994) observed in the rat brain.

The functional characterization of the actions of SR 142948A in the central nervous system showed that this compound, like the first-generation neurotensin receptor antagonist SR 48692, antagonizes the turning behavior induced by intrastriatal injection of neurotensin in mice as well as acetylcholine release evoked by neurotensin in the striatum (Gully et al., 1993, 1997). Neither compound modified dopamine release in the nucleus accumbens after injection of neurotensin into the ventral tegmental area (Steinberg et al., 1994; Gully et al., 1997). However, unlike SR 48692, SR 142948A blocked the hypothermia and analgesia induced by central injection of neurotensin in rodents (Gully et al., 1997). These results suggest that SR 142948A may interact with a neurotensin receptor subtype which is not blocked by SR 48692. It would be interesting to assess the potential involvement of the recently identified neurotensin $NT_2$ receptor in the mediation of the hypothermia and anti-nociceptive effects induced by neurotensin.

In conclusion, $[^3H]SR$ 142948A represents a new potent nonpeptide antagonist radioligand specific for neurotensin receptors. The compound binds to rat neurotensin $NT_1$ and $NT_2$ receptors with nanomolar affinity, close to that of the

natural ligand neurotensin. The ligand previously described, [$^3$H]SR 48692, exhibits a higher affinity for NT$_1$ than for NT$_2$ receptors and has a low ratio of total binding to nonspecific binding in rat brain membranes and tissue sections, which limits its utility. Consequently, the availability of [$^3$H]SR 142948A provides a valuable tool for the study of neurotensin receptors in the central nervous system.

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