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Presence of NK_2 binding sites in the rat brain

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Abstract

Attempts were made to label tachykinin NK_2 binding sites in the adult rat brain using [^{125}I]neurokinin A (NKA) as ligand in the presence of NK_1 and NK_3 agonist or antagonist to avoid labelling of NK_1 and NK_3 binding sites, respectively. A high-affinity, specifically NK_2-sensitive, [^{125}I]NKA-binding, temperature-dependent, reversible, sensitive to GTPγS and correspondence to a single population of binding sites (K_D and B_{max} values: 2.2 nM and 7.3 fmol/mg protein) was demonstrated on hippocampal membranes. Competition studies performed with tachykinins and tachykinin-related compounds indicated that the pharmacological properties of these NK_2-sensitive [^{125}I]NKA binding sites were identical to those identified in the rat urinary bladder and duodenum. NKA, neuropeptide K, and neuropeptide γ, as well as the potent and selective NK_2 antagonists SR 144190, SR 48968 and MEN 10627, presented a nanomolar affinity for these sites. The regional distribution of these NK_2-sensitive [^{125}I]NKA binding sites differs markedly from those of NK_1 and NK_3 binding sites, with the largest labeling being found in the hippocampus, the thalamus and the septum. Binding in other brain structures was low or negligible. A preliminary autoradiographic analysis confirmed [^{125}I]NKA selective binding in hippocampal CA1 and CA3 areas, particularly, and in several thalamic nuclei.

Keywords: hippocampus, (2-[^{125}I]iodohistidyl^1) neurokinin A, NK_2 binding sites, rat brain, tachykinin receptors, thalamus.

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Three types of tachykinin receptors have been cloned, the NK_1, NK_2 and NK_3 receptors whose preferential endogenous ligands are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), respectively. The molecular structure of each of these receptors differs slightly among species, particularly between the rat and the guinea-pig, and the structures of human receptors are closely similar to those of the guinea-pig (Nakanishi 1991; Krause *et al*. 1994). In addition, as suggested by binding studies and biological responses, there is evidence for the existence of different subtypes or conformers of these receptors, particularly in the case of NK_1 receptors (Petitet *et al*. 1992; Maggi and Schwartz 1997; Beaujouan *et al*. 2000).

Although the presence of tachykinin NK_1 and NK_3 receptors is well established in both the CNS and peripheral tissues, this is not the case for NK_2 receptors. NK_2 binding sites were first identified in some peripheral tissues such as the rat duodenum and urinary bladder using [^{125}I]Bolton-Hunter NKA (Buck *et al*. 1984; Burcher and Buck 1986) and then [^{3}H]NKA (Bergström *et al*. 1987a) as ligands. However, in spite of the numerous efforts of several groups, binding sites for central NK_2 receptors were not clearly identified in the adult brain. In fact, both [^{125}I]Bolton-Hunter NKA and [^{3}H]NKA were used without success in binding studies performed on brain membranes and, in addition, were shown to have a poor selectivity in autoradiographic investigations (Lee *et al*. 1986; Bergström *et al*. 1987a; Saffroy *et al*. 1988; Mantyh *et al*. 1989; Dietl and Palacios 1991). Indeed, the addition of the [^{127}I]Bolton-Hunter derivative to the NKA molecule enhances the ligand affinity for NK_3 binding sites (Lee *et al*. 1986; Bergström *et al*. 1987b). Moreover, we have recently shown that NKA has also a high affinity for central subtypes of NK_1 binding sites (Beaujouan *et al*. 2000). Unsuccessful attempts to label central NK_2 binding sites in the adult rat were also made on either membranes or brain sections with other ligands such as the selective agonist [^{125}I][Lys^5,Tyr(I$_2$)^7, MeLeu^9,Nle^{10}]NKA(4–10) or the NK_2 receptor antagonists [^{3}H]GR 100679, [^{125}I]BH-MEN 10376 and [^{3}H]SR 48968 since specific binding was either absent or very low

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Abbreviations used: BSA, bovine serum albumin; [^{125}I]NKA, (2-[^{125}I]iodohistidyl^1) neurokinin A; NKB, neurokinin B; NPK, neuropeptide K; NPγ, neuropeptide γ; SP, substance P.

(Badgery-Parker *et al*. 1993; Hagan *et al*. 1993; Humpel and Saria 1993; Stratton *et al*. 1996). Nevertheless, NK_2 binding sites could be revealed in some brain structures of the newborn rat thanks to [^{3}H]GR 100679 (Hagan *et al*. 1993). A specific binding of [^{125}I]NKA has been observed on brain membranes or autoradiograms, but this ligand labeled central subtypes of NK_1 binding sites and NK_3 binding sites (Saffroy *et al*. 1988; Quirion *et al*. 1991; Beaujouan *et al*. 2000). However, binding sites were found in layer 6 of the cerebral cortex of 6-day postnatal animals, but not in the adult rat, suggesting for the first time a labeling of NK_2 binding sites with [^{125}I]NKA, this cortical layer being devoid of NK_1 and NK_3 binding sites (Dam *et al*. 1988). Interestingly, some indications for the presence of central NK_2 binding sites were also obtained using [^{125}I]NPγ as radioligand (Dam *et al*. 1990) but, unfortunately, [^{125}I]NPγ also labels NK_1 binding sites (Watling *et al*. 1993). This is in agreement with our recent observations which indicate that NPγ, as well as NKA and NPK, exhibit a high affinity for central subtypes of NK_1 binding sites (Beaujouan *et al*. 2000).

Tachykinin NK_2 receptors have been cloned in peripheral tissues from the rat (Sasai and Nakanishi 1989). Although the presence of mRNA for these receptors was clearly shown in these tissues, surprisingly, mRNAs for NK_2 receptors were detected in only very limited amounts in the CNS (Tsuchida *et al*. 1990; Takeda and Krause 1991; Whitty *et al*. 1995). Nevertheless, the occurrence of NK_2 receptor mRNA has recently been revealed in the rat septal area thanks to RT-PCR analysis, but its level of expression is considerably lower than in peripheral tissues (Steinberg *et al*. 1998a). In addition, using a fluorescently tagged NKA, evidence for the presence of NK_2 receptors was obtained in a subset of neurons from the medial septal-diagonal band complex (Steinberg *et al*. 1998a). Although in one study, immunostaining of NK_2 receptors could be shown in astrocytes from the rat spinal cord (Zerari *et al*. 1998), the presence of the NK_2 receptor protein in the rat brain has not yet been demonstrated using this approach.

To our knowledge, there is yet no clear cellular or tissue evidence for an effect of NKA on intracellular signalling processes (second messengers) mediated by NK_2 receptors in brain preparations. Electrophysiological studies have been performed with tachykinins in different brain areas from either the rat or the guinea pig, but only few of them have described a response evoked by NKA or a NK_2 agonist. In fact, divergent results were obtained in studies carried out on dopaminergic neurons from the rat mesencephalon (Innis *et al*. 1985; Keegan *et al*. 1992; Overton *et al*. 1992; Seabrook *et al*. 1995), and the use of selective antagonists for each class of tachykinin receptor is absolutely required for the precise identification of the receptors implicated in the responses evoked by NKA or NK_2 agonists. For example, in one study (Maubach and Jones 1997), responses induced by either NKA or septide in neurons from the rat NST or DMNV were shown to be antagonized by an NK_1 antagonist, while in another study performed on neurons from the dorsal motor nucleus of the vagus (DMNV) (Martini-Luccarini *et al*. 1996), the depolarizing effect of NKA was partially abolished by a NK_2 antagonist suggesting that NKA may act on both NK_1 receptors from the 'septide type' and NK_2 receptors in the CNS. Contrasting with most of these results, clear biochemical or behavioural responses evoked by NK_2 agonists and blocked by selective NK_2 antagonists have been shown in rodent brains (Tschöpe *et al*. 1992; Walsh *et al*. 1992; Hagan *et al*. 1993; Picard *et al*. 1994; Emonds-Alt *et al*. 1997; Steinberg *et al*. 1998a,b). In fact, this was first observed in our laboratory, since the selective NK_2 agonist [Lys^5,MeLeu^9,Nle^{10}]NKA(4–10) was found to stimulate the release of dopamine in the matrix of the rat striatum and this response was blocked by the potent selective NK_2 non-peptide antagonist SR 48968 (Tremblay *et al*. 1992).

Altogether, these considerations led us in the present study to look again for the presence of NK_2 binding sites in the rat brain. From the observations described above, [^{125}I]NPγ and [^{125}I]NKA were the best candidates as radioligands for this study. However, [^{125}I]NKA was selected since the iodination of one histidyl residue is easier to control than the iodination of the three histidyl residues of NPγ. [^{125}I]NKA exhibits a high specific radioactivity but, as previously described, this ligand is unselective. Therefore, [^{125}I]NKA was used in the presence of selective agonists or antagonists of NK_1 and NK_3 binding sites. In addition, likely due to the presence of a reduced density of NK_2 receptors in the brain, membrane preparations and binding conditions similar to those chosen for the identification of central subtypes of NK_1 binding sites with [^{125}I]NKA (Beaujouan *et al*. 2000) were used in our study. Finally, care was also made to compare the pharmacological properties of central and peripheral NK_2 binding sites.

Materials and methods

Ligands and drugs

[^{125}I]NKA (2000 Ci/mmol) and [^{3}H]NKA (93.8 Ci/mmol) were from Amersham Corp. (Buckinghamshire, UK). [Lys^5,MeLeu^9,Nle^{10}]NKA(4–10), NKA(4–10), ALIE-124 and [Apa9–10]SP were synthesized by S. Lavielle and G. Chassaing (Laboratoire de Chimie Organique Biologique, UMR CNRS 7613, Université P. et M. Curie, Paris, France). SR 48965, SR 48968, SR 140333, SR 142801 and SR 144190 were kindly provided by X. Emonds-Alt (Sanofi, Montpelier, France); RP 67580 and CP 96345 by J. C. Blanchard (Rhône Poulenc-Rorer, France); MEN 10627 and MEN 11420 by L. Quartara (Menarini Ricerche, Florence, Italy). [Sar^9,Met(O$_2$)^{11}]SP was from Genosys (Cambridge, UK). [Pro^9]SP, senktide, NPK, GR 64349, GR 82334, GR 87389, GR 94800, R 396 and AcArgSeptide were from Neosystem (Strasbourg, France). NKA, [Lys^5]NKA(4–10), septide, [MePhe^7]NKB and scyliorhinin I

were purchased from Bachem (Voisins-le-Bretoneuse, France). All other peptides used were purchased from Peninsula laboratories (Merseyside, UK).

Membrane preparations

Male Sprague–Dawley rats (200–250 g) (Charles River, Cléon, France) were housed in groups of five with food and water *ad libitum* in a room maintained at 22°C with 12-h/12-h light/dark cycle. Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals established by the National Institute of Health and with the European Community Council Directive 86/609 EEC. Membranes from several brain structures and the urinary bladder were prepared as previously described (Beaujouan *et al*. 1999). The final membrane pellet was resuspended in the incubation medium consisting of Tris-HCl buffer, 50 mM, pH 7.4, enriched in $MnCl_2$ (3 mM), bovine serum albumin (BSA) 0.1% (Calbiochem, San Diego, CA, USA), bacitracin (200 μg/mL) (Janssen, Geel, Belgium), leupeptin (4 μg/mL) (Sigma, St Louis, MO, USA), chymostatin (2 μg/mL) (Sigma, St Louis, MO, USA), captopril (2 μM) (generous gift from Squibb Laboratory, Neuilly/Seine, France), amastatin (2 μM) (Sigma, St Louis, MO, USA) and thiorphan (10 μM) (Sigma). Membranes from smooth muscles of the rat duodenum were prepared as previously described (Bergström *et al*. 1987a).

[^{125}I]NKA binding assays

Membranes from various brain structures (60 μL, about 400 μg of protein per assay) were incubated for 45 min with [^{125}I]NKA (2000 Ci/mmol, ~70 pM) at 20°C (final volume 200 μL). Binding assays were performed in the presence of SR 140333 (0.1 μM), a selective NK_1 antagonist, and senktide (1 μM), a selective NK_3 agonist, to prevent [^{125}I]NKA binding to NK_1 and NK_3 binding sites, respectively. The duration of the incubation corresponds to the time at which binding equilibrium was reached as determined from kinetic experiments performed in identical conditions. Incubations were stopped by filtration through Whatman GF/C filters (25 mm of diameter) pretreated for 3–4 h at 4°C with 0.1% polyethylenimine (Sigma) using a J.S.I. Multividor apparatus. Filters were then washed three times at 4°C with 3 mL of Tris-HCl buffer (50 mM, pH 7.4) containing $MnCl_2$ (3 mM) and BSA (0.1%). Pellet-bound radioactivity was estimated in a Beckman liquid spectrometer using 3 mL of OptiPhase 'HiSafe'3 (Wallac) after a 3-h equilibrium period. When membranes of the urinary bladder were used, binding assays were performed as just described with minor modifications. Membranes (60 μL, about 50 μg of protein per assay) were incubated at 20°C for 45 min with ([^{125}I]NKA ~35 pM) in the presence of [Pro^5]SP (1 μM), a selective NK_1 agonist.

[^{3}H]NKA binding assays

Binding assays were performed as previously described (Bergström *et al*. 1987a) with minor modifications. Membranes from duodenal smooth muscles (~120 μg protein per assay) were incubated for 25 min at 20°C with [^{3}H]NKA.

Autoradiographic experiments

Adjacent coronal sections of male Sprague–Dawley rats (200–250 g) (Charles River, Cléon, France) were prepared and binding experiments were essentially performed as previously described (Saffroy *et al*. 1988) with minor modifications. Tissue sections (16 μm) were preincubated first at room temperature for 15 min in HEPES (20 mM, pH 7.4) containing KCl (300 mM) and EDTA (10 mM), and washed for 15 min in Tris-HCl (50 mM, pH 7.4) containing BSA (0.02%). Brain sections were then incubated for 90 min at room temperature in Tris-HCl (50 mM, pH 7.4) containing BSA (0.02%), $MnCl_2$ (3 mM), bacitracin (4 mg%), leupeptin (0.4 mg%) and chymostatin (0.2 mg%). [^{125}I]NKA was added (220 000 cpm/mL, 50 pM) in the presence of senktide (1 μM) and SR 140333 (0.1 μM) or senktide (1 μM) and [Pro^9]SP (1 μM) and in the presence or absence of either NKA (1 μM) or SR 48968 (0.1 μM). At the end of the incubation, tissue sections were washed (5 × 30 s), in ice-cold Tris-HCl (50 mM, pH 7.4) containing BSA (0.02%) and for 30 s with H_2O. Finally, sections were rapidly dried under a stream of air and slide-mounted tissue sections were exposed to Amersham tritium-sensitive Hyperfilm for 7 weeks. Films were developed (5 min) at 18°C using Kodak D19 developer. Sections were then stained with cresyl violet for histological identification of brain structures according to the atlas of Paxinos and Watson (1997). Autoradiograms were analyzed using an image analyzer, NIH IMAGE equipment.

HPLC analysis

The identity of the radioactive material present in the supernatant at the end of the incubation was checked to verify that radioligands were not degraded by peptidases. For this purpose, high pressure liquid chromatography was performed using C18 μBondapak columns (Waters Instruments, Rochester, MN, USA) and methanol/ammonium acetate (50 mM, pH 7.4) (1/1, v/v) as solvent.

Data analysis

Data were expressed as means ± SEM. Statistical analysis were performed using ANOVA followed by the Student–Newman–Keuls' test. p-values of < 0.05 were considered significant. Sigmoid equilibrium competition curves were analyzed using GraphPad PRISM Software, Inc. (San Diego, CA, USA). Curves were fitted using SIGMAPLOT software (Jandel Scientific, Erkrath, Germany). K_i values were calculated from the equation: $K_i = IC_{50}/[1 + ([\text{radioligand}]/K_D)]$.

Results

Binding of [^{125}I]NKA to rat hippocampal membranes

Preliminary experiments were first performed on membranes from different brain structures with [^{125}I]NKA in the presence of SR 140333 (0.1 μM, NK_1 antagonist) and senktide (1 μM, NK_3 agonist) to completely avoid labeling of NK_1 and NK_3 binding sites, respectively. The most important specific labeling was found in the hippocampus. Total and non-specific [^{125}I]NKA bindings on hippocampal membranes increased linearly with protein concentration up to 600 μg/200 μL assay. Routinely, a protein concentration of 400 μg/200 μL was used and incubations were carried out at 20°C for 45-min with 0.08 nM [^{125}I]NKA in the presence or absence of NKA (1 μM). Under these conditions, specific [^{125}I]NKA binding represented 50% of total binding and 0.75% of total radioactivity added to the incubation medium. As revealed by HPLC analysis, 77% of the radioactivity corresponded to [^{125}I]NKA after a 45-min

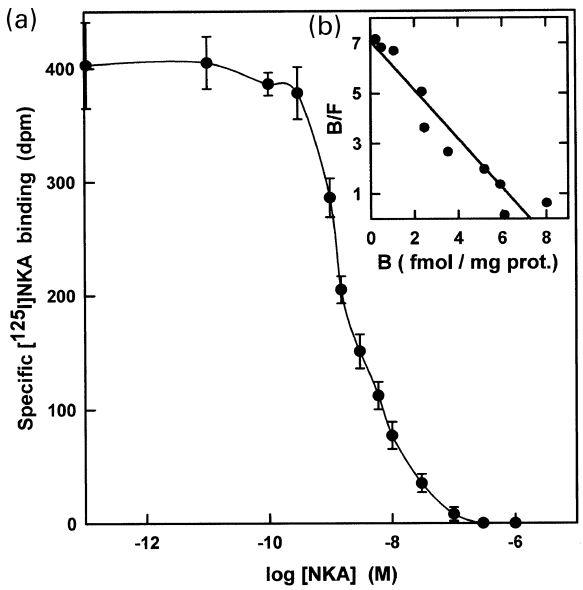


Fig. 1 Competitive inhibition by NKA of specific [^{125}I]NKA binding to membranes from the hippocampus. (a) Hippocampal membranes were incubated with [^{125}I]NKA in the presence of 1 μM senktide and 0.1 μM SR 140333 and increasing concentrations of NKA as described in Materials and methods. Results are the mean ± SEM of data obtained in four independent experiments performed in triplicate. (b) Scatchard plot of specific [^{125}I]NKA binding data.

incubation at 20°C carried out with the cocktail of peptidase inhibitors used in our binding conditions (see Materials and methods). This indicates a minor degradation of the labeled peptide.

Characteristics of [^{125}I]NKA binding

The binding of [^{125}I]NKA to hippocampal membranes reached equilibrium within the 45-min incubation at 20°C (calculated $t_{1/2}$ association: 14 min). Specific [^{125}I]NKA binding was temperature-dependent and negligible when the incubation was carried out at 4°C. At 20°C, specific [^{125}I]NKA binding was reversible, since a slow exponential decay of bound [^{125}I]NKA was observed following the addition of 1 μM NKA to the incubation medium after a 45-min incubation period (calculated $t_{1/2}$ dissociation time: 162 min). As achieved in other binding studies performed with [^{125}I]NKA (Ciucci *et al*. 1998; Beaujouan *et al*. 2000), competition experiments were made with NKA to determine apparent K_D and B_{max} values (2.2 ± 0.4 nM and 7.3 ± 0.8 fmol/mg protein, respectively, *n*H 1.04 ± 0.11, means of data obtained in four distinct experiments) (Fig. 1).

Competitive studies with tachykinins and tachykinin-related compounds

When potent tachykinins and tachykinin-related compounds inhibited competitively and in a concentration-dependent manner, specific [^{125}I]NKA binding to hippocampal membranes allowed the determination of the K_i values. As expected, NKA (K_i 1.8 ± 0.1 nM), NPK and NPγ inhibited with high affinity this binding. Selective NK_2 agonists and antagonists were also potent inhibitors. Rank orders of affinity were NPγ = NPK = NKA > NKB > SP for endogenous mammalian tachykinins, [Lys^5,MeLeu^9, Nle^{10}]NKA(4–10) > [βAla^8]NKA(4–10) for tachykinin agonists, and finally SR 144190 = MEN 10627 = SR 48968 = MEN 11420 > GR 94800 > GR 87389 > R 396 > MEN 10376 > MEN 10207 for non-peptide and peptide tachykinin antagonists. The inhibitory effect of SR 48968 was stereoselective since the (*R*)-enantiomer, SR 48965, was about 1900-fold less potent. As expected, selective NK_1 and NK_3 agonists and antagonists were without effect or had a minor affinity for [^{125}I]NKA binding sites. This was also the case for SP-related compounds such as ALIE-124, septide and SP(6–11), characterized by their high affinity for 'septide-sensitive' binding sites. Scyliorhinin I and kassinin were the most potent competitors among non-mammalian tachykinins (Table 1 and Fig. 2). In most cases, analysis of experimental curves suggested the presence of an homogeneous population of [^{125}I]NKA binding sites. However, a biphasic displacement of [^{125}I]NKA binding was observed in the presence of increasing concentrations of [βAla^8]NKA(4–10). The fit was statistically significant for a two-site model. Therefore, two K_i values corresponding to 8.0 nM and 269 nM and representing 43.5% and 56.5% of the binding sites, respectively, were calculated for this selective NK_2 agonist.

Similarity of the pharmacological profiles of central and peripheral NK_2 binding sites

As illustrated in Table 1 and Fig. 3, each tachykinin compound inhibited, with a similar potency, NK_2-sensitive [^{125}I]NKA binding to membranes from the hippocampus and the urinary bladder (hippocampus/urinary bladder: $s = 0.92$, $r = 0.97$). This was also the case when the potency of each of these compounds to inhibit NK_2-sensitive [^{125}I]NKA binding to membranes from the hippocampus was compared with its potency to inhibit [^{3}H]NKA binding to membranes from the duodenal smooth muscle (hippocampus/duodenal smooth muscle: $s = 0.79$, $r = 0.96$, data not shown).

In contrast with observations with hippocampal membranes, the NK_2 agonist [βAla^8]NKA(4–10) did not allow distinction between the two populations of NK_2-sensitive [^{125}I]NKA binding sites on urinary bladder membranes. However, biphasic displacements of [^{125}I]NKA binding were observed in the urinary bladder with either SP(6–11) (K_i 268 and 5470 nM; 42%/58%), eledoisin (K_i 1.5 and 86 nM; 15%/85%), physalaemin (K_i 186 and 5540 nM; 40%/60%), SP (K_i 186 and 5060 nM; 63%/37%), GR 64349 (K_i 1.9 and 24 nM; 64%/36%), with fits being statistically significant for a two-site model.

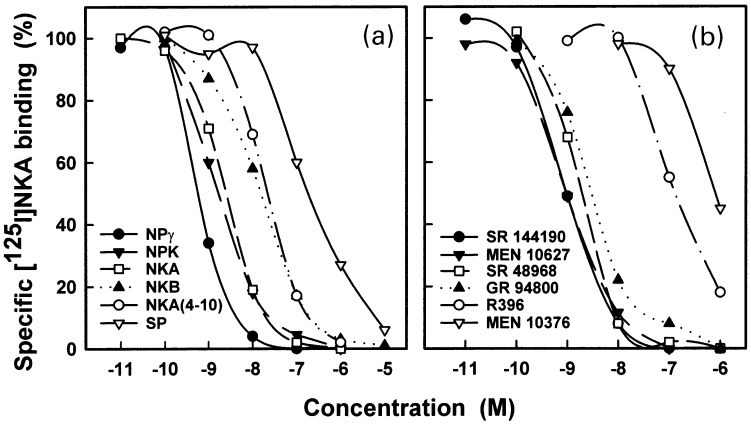


Fig. 2 Competitive inhibition of NK_2-sensitive [^{125}I]NKA binding by endogenous tachykinins and selective NK_2 antagonists in the hippocampus. Competition studies with increasing concentrations of endogenous tachykinins (a) and selective NK_2 antagonists (b) were performed in the presence of 1 μM senktide (NK_3 agonist) and 0.1 μM SR 140333 (NK_1 antagonist) using [^{125}I]NKA and membranes from the rat hippocampus as indicated in Materials and methods. Results were expressed as percentage of corresponding specific [^{125}I]NKA binding. Each point is the mean of data obtained in between three and seven experiments, each value being determined in triplicate.

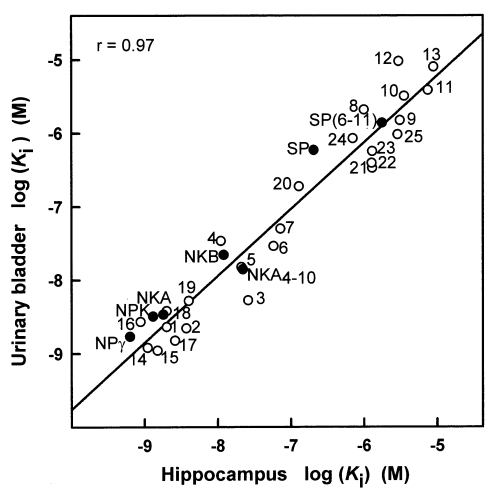
As expected, marked differences were found between the pharmacological profiles of central (or peripheral) NK_2-sensitive [^{125}I]NKA binding sites and those of non-classical central NK_1 binding sites that were recently identified with [^{125}I]NKA in the presence of SR 48968 and senktide to avoid binding to NK_2 and NK_3 binding sites (Beaujouan *et al*. 2000; data not shown).

Effects of GTPγS on hippocampal NK_2-sensitive [^{125}I]NKA binding

Supporting a coupling of NK_2 receptors to G proteins, GTPγS inhibited competitively and in a concentration-dependent manner specific [^{125}I]NKA binding to hippocampal membranes (K_i = 1230 ± 400 nM), this inhibition being complete at high concentration. GTPγS did not significantly affect the K_i of NKA [K_i(NKA) 2.43 ± 0.24 nM; K_i(NKA + 5 μM GTPγS) 3.25 ± 0.89 nM] when using a concentration of 5 μM which led to more than 50% inhibition of [^{125}I]NKA-specific binding.

Regional distribution of NK_2-sensitive [^{125}I]NKA binding sites

Binding studies were performed on membranes from different brain structures using two experimental procedures. Experiments were first performed in the presence of 0.1 μM SR 140333 + 1 μM senktide and specific [^{125}I]NKA binding was determined by adding 1 μM NKA. Other binding assays were carried out in the presence of 1 μM senktide and specific [^{125}I]NKA binding was determined by adding 0.1 μM SR 48968. At this concentration, SR 48968 affects neither NK_1-sensitive [^{125}I]NKA binding nor specific [^{3}H]senktide binding to rat cortical membranes (Petitet *et al*. 1993; Beaujouan *et al*. 2000). Similar results were obtained with these two procedures (no significant difference according to the ANOVA followed by Student–Newman–Keuls' test). Indeed, in both cases, specific [^{125}I]NKA binding was found to be higher in the hippocampus than in the thalamus or the septum, in which specific binding represented about 30–40% that observed in the hippocampus.



1 [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)
2 [Lys^5]NKA(4-10)
3 GR 64349
4 scyliorhinin I
5 kassinin
6 [βAla^8]NKA(4-10)
7 eledoisin
8 SP(5-11)
9 physalaemin
10 Ac-Arg-septide
11 [MePhe^7]NKB
12 [Sar^{9}Met(O$_2$)^{11}]SP
13 scyliorhinin II
14 MEN 10627
15 SR 48968
16 SR 144190
17 GR 94800
18 MEN 11420
19 GR 87389
20 R396
21 MEN 10207
22 SR 142806
23 SR 142801
24 MEN 10376
25 SR 48965

Fig. 3 Correlation between log of K_i values (M) of tachykinins and tachykinin related compounds for central and peripheral NK_2 binding sites. K_i values of tachykinins and tachykinin-related compounds for inhibiting the specific NK_2-sensitive [^{125}I]NKA binding to membranes from the rat hippocampus (in the presence of 0.1 μM SR 140333 and 1 μM senktide) and from the rat urinary bladder (in the presence of 1 μM [Pro^9]SP) were used to establish this correlation between central and peripheral NK_2 binding sites. The correlation line was fitted using the SIGMAPLOT software. ●, main tachykinins or their C-terminal fragments. ○, numbers corresponding to compounds indicated on the right side of the figure.

Table 1 Respective K_i values of tachykinin compounds for inhibiting NK_2-sensitive [^{125}I]NKA binding in the hippocampus and urinary bladder and [^{3}H]NKA binding in the duodenal smooth muscle

Compounds		Hippocampus K_i (nM)	Urinary bladder K_i (nM)	Duodenum K_i (nM)
NK_2 agonists				
	NKA	1.8	3.4	7.4*
	NPK	1.3	3.2	1.9
	NPγ	0.63	1.7	2.3
	NKA(4–10)	22	14	9.5*
	[Lys^{5}MeLeu^{9}Nle^{10}]NKA(4–10)	2.0	2.3	5.7
	[Lys^5]NKA(4–10)	3.7	2.2	5.6
	GR 64349	26	5.3	13
	[βAla^8]NKA(4–10)	58	29	23
NK_2 antagonists				
	SR 144190	0.88	2.7	6.1
	MEN 10627	1.1	1.2	3.3
	SR 48968	1.5	1.1	2.9
	MEN 11420	2.0	3.8	5.6
	GR 94800	2.6	1.5	4.6
	GR 87389	4.0	5.2	14
	R 396	129	189	292
	MEN 10376	703	850	2340
	MEN 10207	1290	336	1050
	SR 48965	2875	960	1170
NK_1 agonists				
	SP	205	590	206*
	SP(6–11)	1560	1385	159
	SP(5–11)	996	2090	1610
	[Pro^9]SP	3900		$>$ 10 000*
	[Sar^{9}Met(O$_2$)11]SP	2960	9520	552
	[Apa$^{9-10}$]SP	$>$ 10 000	$>$ 10 000	$>$ 10 000
	Septide	$>$ 10 000	$>$ 10 000	$>$ 10 000
	AcArgSeptide	3530	3210	2620
	ALIE-124	11 700	$>$ 10 000	2670
NK_1 antagonists				
	RP 67580	$>$ 10 000	$>$ 10 000	$>$ 10 000
	SR 140333		2240	1250
	CP 96345	$>$ 10 000	$>$ 10 000	$>$ 10 000
	GR 82334	$>$ 10 000	22 300	$>$ 10 000
NK_3 agonists				
	NKB	12	22	33*
	Senktide		$>$ 10 000	$>$ 10 000
	[MePhe^7]NKB	7440	3860	6560
NK_3 antagonist				
	SR 142801	1290	568	2380
Non-mammalian tachykinins				
	Scyliorhinin I	11	34	22
	Kassinin	21	15	25*
	Eledoisin	72	50	27*
	Physalaemin	3090	1495	936*
	Scyliorhinin II	8860	7990	1630

K_i values (as determined in competitive studies) of various tachykinin compounds for inhibiting NK_2-sensitive [^{125}I]NKA binding to membranes from the rat hippocampus (in the presence of 0.1 μM SR 140333 and 1 μM senktide) and urinary bladder (in the presence of 1 μM [Pro^9]SP) were compared with K_i values of these compounds for inhibiting [^{3}H]NKA binding to membranes of the rat duodenal smooth muscle. Binding assays were performed as described in Materials and methods. K_i values were calculated from IC_{50} values corresponding to the mean $\pm$ SEM of data obtained in between three and seven independent experiments each value being determined in triplicate. Standard deviation of binding assays performed in the hippocampus and urinary bladder did not exceed 29%, and 28% of the mean, respectively. *K_i values in the duodenum were taken from Bergström *et al.* (1987a) while additional experiments were made in the same experimental conditions to complete the table, standard deviation did not exceed 42% of the mean. SR 48965 is the (*R*)-enantiomer of the NK_2 antagonist SR 48968.

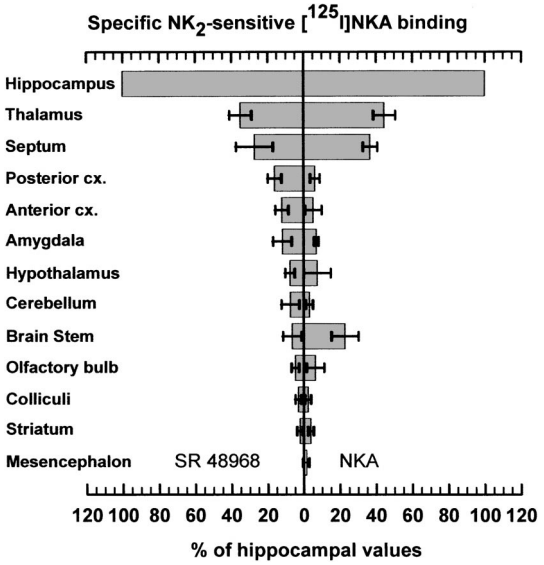


Fig. 4 Regional distribution of specific NK_2-sensitive [^{125}I]NKA binding sites in the rat brain. For each structure, membranes were prepared from pooled tissues of 18 animals. (a) Specific binding of [^{125}I]NKA (displaced by 0.1 μM SR 48968) was determined following a 45-min incubation at 20°C performed in the presence of 1 μM senktide. (b) Specific binding of [^{125}I]NKA (displaced by 1 μM NKA) was performed in identical conditions but in the presence of 1 μM senktide and 0.1 μM SR 140333. Results are expressed in percentage of hippocampal values and are the means ± SEM from data obtained in between three and nine independent experiments performed in triplicate. Hippocampal values obtained in experiments performed with either SR 48968 or NKA are 697 ± 56 ($n = 9$) and 516 ± 91 ($n = 6$) cpm/mg protein, respectively.

A much lower but significant specific NK_2-sensitive [^{125}I]NKA binding was observed in the posterior cortex and the amygdala while low or negligible binding occurred in other structures (Fig. 4).

Autoradiographic localization of NK_2-sensitive binding sites

Adjacent coronal sections performed at the level of the dorsal hippocampus/thalamus were incubated with [^{125}I]NKA in the presence of either 0.1 μM SR 140333 and 1 μM senktide or 1 μM [Pro^9]SP and 1 μM senktide (four experiments in each condition). Autoradiograms were prepared as described in Materials and methods. Brain sections were also used to determine non-specific binding with either 1 μM NKA or 0.1 μM SR 48968. Except in the cerebral cortex in which non-specific binding corresponded to 23% of total binding; non-specific binding was negligible in all areas. In both experimental conditions and in agreement with binding data obtained with membranes, the highest labeling was observed in the hippocampus. More precisely, labeling was important in the stratum radiatum (Rad) and less important in the oriens layer (Or) of the CA1 and CA3 fields of the Ammon's horn. In contrast, the polymorph layer of the dentate gyrus (PoDG) and the dentate gyrus (DG) were devoid of labeling. Most thalamic nuclei were labeled, including particularly the mediodorsal (MD, except in its central part), posterior nuclear group (Po), rhomboid (Rh) and ventromedial (VM) thalamic nuclei. In contrast, in the same sections, other brain structures such as the habenula, the hypothalamus and the amygdala were either weakly labeled or completely devoid of labeling (Fig. 5).

Discussion

Several observations of this study indicate that [^{125}I]NKA is a suitable ligand for labeling NK_2 binding sites from either homogenates or sections of the rat brain. Previous unsuccessful attempts of several groups may result mainly from the lack of selectivity of the radioligands used, combined with the rather limited density of central NK_2 binding sites. The metabolic instability or, in some cases, the low specific activity of the ligands could also be important limiting factors in the identification of these binding sites. In addition, the relative instability (state modifications) of accessible high affinity binding sites may also be critical. In this context, some technical aspects of our study should be underlined. Indeed, experiments should be performed with strict respect to timing and steps carried out in the presence of appropriate NK_1 and NK_3 agonists or antagonists and peptidase inhibitors, chosen following careful control of the radioligand state at the end of the incubation. These conditions were those which previously allowed us to detect the low amounts of NK_1 binding site subtypes in large quantities of homogenates prepared from distinct brain structures of the rat (Beaujouan *et al*. 2000).

In these precise binding conditions, according to kinetic studies performed on hippocampal membranes, [^{125}I]NKA was found to bind reversibly and with high affinity to a single population of non-interacting sites. The density of these [^{125}I]NKA binding sites is relatively low (7.3 fmol/mg of protein) when compared with those of either NK_2 binding sites in some peripheral tissues (Bergström *et al*. 1987a; Emonds-Alt *et al*. 1993; Matuszek *et al*. 1998) or classical NK_1 and NK_3 binding sites in the brain (Torrens *et al*. 1983; Beaujouan *et al*. 1984). In some way, this observation is reminiscent of results obtained in RT-PCR experiments which indicated the presence of low amounts of NK_2 receptor mRNA in the septum when compared with their large amounts found in peripheral tissues such as the colon (Steinberg *et al*. 1998a). This could explain the unsuccessful results of previous *in situ* hybridization studies. Similar affinities of natural tachykinins and tachykinin-related compounds were found for central NK_2-sensitive [^{125}I]NKA binding sites and peripheral NK_2 sites labeled with either [^{125}I]NKA or [^{3}H]NKA. Indeed, NKA, NPK, NPγ and selective NK_2 agonists and antagonists, which present a high

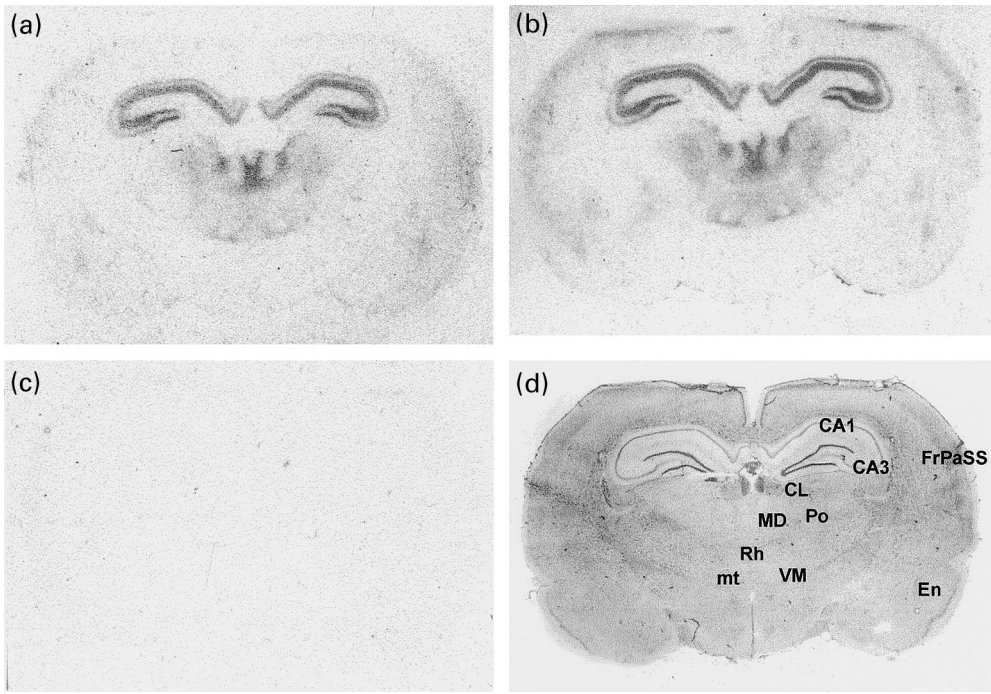


Fig. 5 Autoradiographic localization of NK_2-sensitive [^{125}I]NKA binding sites in the rat brain. Adjacent coronal sections made at the level of the dorsal hippocampus/thalamus were incubated with [^{125}I]NKA in the presence of 1 μM senktide and either 0.1 μM SR 140333 (a) or 1 μM [Pro^9]SP (b). Non-specific binding (c) observed in the experiment performed with senktide, SR 140333 and SR 48968. Similar autoradiographs were obtained for non-specific binding when either [Pro^9]SP was used instead of SR140333 or when NKA was used instead of SR 48968. Corresponding brain section stained with cresyl violet (d). CA1, field CA1 of Ammon's horn; CA3, field CA3 of Ammon's horn; CL, centrolateral thalamic nucleus; En, endopiriform nucleus; FrPaSS, frontoparietal cortex, somatosensory area; MD, mediodorsal thalamic nucleus; mt, mammillothalamic tract; Po, posterior thalamic nuclear group; Rh, rhomboid thalamic nucleus; VM, ventromedial thalamic nucleus.

affinity and potent activity on peripheral NK_2 receptors, inhibited with high affinity specific [^{125}I]NKA binding to hippocampal membranes. As expected, SP, NKB and selective NK_1 and NK_3 agonists or antagonists exhibited only low or no affinity for specific [^{125}I]NKA binding sites. The pharmacological profile of NK_2 binding sites in the hippocampus was closely similar to that found for these binding sites in either the urinary bladder or the duodenum. Altogether, these pharmacological results agree with those of other workers who characterized NK_2 receptors on either CHO and COS cells transfected with the cDNA for the rat tachykinin NK_2 receptor or membranes from the rat urinary bladder, colon, fundus or vas deferens using [^{125}I]NKA or [^{125}I][Lys^5,MeLeu^9,Nle^{10}]NKA(4–10), respectively (Ingi *et al*. 1991; Cascieri *et al*. 1992; Matuszek *et al*. 1998).

These observations provide strong evidence for the homogeneity of tachykinin NK_2 receptors in rat tissues. However, competitive studies made with membranes from the rat small intestine with [^{125}I]NKA, [βAla^8]NKA(4–10) and GR 64349 have suggested the presence of two affinity states or conformers for NK_2 receptors (Brown *et al*. 1992; Renzetti *et al*. 1997). Interestingly, while [βAla^8]NKA(4–10) was found to bind to a single population of binding sites in the rat urinary bladder, high and low affinity binding sites for this selective NK_2 agonist were detected in hippocampal membranes. This result was not confirmed with GR 64349 since the displacement curve was monophasic in hippocampal membranes.

Tachykinin receptors are coupled to G proteins. According to studies performed on peripheral tissues, in most cases, selective NK_1, NK_2 or NK_3 agonists stimulate phosphoinositide hydrolysis (Guard and Watson 1991; Khawaja and Rogers 1996). For instance, in the rat urinary bladder, NK_2 agonists induce a marked response which was selectively blocked by NK_2 antagonists (Torrens *et al*. 1995). NK_2-sensitive [^{125}I]NKA binding sites in the hippocampus are also likely coupled to G proteins, since specific [^{125}I]NKA binding was completely abolished in the presence of GTPγS. Attempts to demonstrate a NK_2 receptor-mediated stimulation of phosphoinositide hydrolysis were unsuccessful (data not shown). This could be due to the rather low density of NK_2 receptors in the hippocampus but it should be recalled that for still non-elucidated reasons, such tachykinin

responses are of low amplitude or even absent in brain structures of the adult rat (Mantyh *et al*. 1984; Hunter *et al*. 1985; Lebrun *et al*. 1993; unpublished observations).

Further demonstrating the specificity of labeling, the regional distribution of NK_2-sensitive [^{125}I]NKA binding sites completely differs from those of NK_1 and NK_3 binding sites (Beaujouan *et al*. 1984; Laufer *et al*. 1986). NK_2 binding sites were found in only a few brain structures including the hippocampus, the septum and the thalamus, a structure poor in NK_1 and NK_3 binding sites when compared with other structures. The occurrence of NK_2 binding sites in the septum agrees with recent observations indicating the presence of NK_2 receptor mRNA and labeling with fluorescently tagged NKA in some neurons of this structure (Steinberg *et al*. 1998a). The occurrence of NK_2-sensitive [^{125}I]NKA binding sites in the hippocampus and the thalamus was confirmed by preliminary autoradiographic data obtained in the presence of senktide and [Pro^9]SP (or SR 140333) to avoid [^{125}I]NKA binding to NK_3 and non-classical NK_1 binding sites, respectively. Other workers who used [^{125}I]NPγ have also attempted to visualize central NK_2 binding sites (Dam *et al*. 1990), but as already indicated, this ligand labels both NK_2 and NK_1 binding sites (Watling *et al*. 1993). However, in agreement with our own observations, the hippocampus and the thalamus were selectively labeled when [^{125}I]NPγ was used in the presence of the selective NK_1 agonist, [Sar^9,Met(O$_2$)^{11}]SP (Watling *et al*. 1993). Nevertheless, while some hippocampal areas are labeled with both [^{125}I]NPγ and [^{125}I]NKA (our study), other areas such as the CA4 field of Ammon's horn and the dentate gyrus are labeled with only [^{125}I]NPγ. Interestingly, in these latter areas, and in contrast with observations in other hippocampal sites, [^{125}I]NPγ labeling appeared to be resistant to the NK_2 antagonist L-659877. As also reported, some central responses induced by local applications of NPγ are resistant to the action of both NK_2 and NK_1 antagonists (Picard and Couture 1996). Finally, the selective NK_2 antagonist [^{3}H]GR 100679 has also been used in autoradiographic studies performed on neonatal and adult rat brains (Hagan *et al*. 1993). Experiments in the adult were not successful due to a high level of non-specific labeling, but specific NK_2 binding sites were detected in the hippocampus and the thalamus of the neonates.

NKA, NPK and NPγ, the preferential endogenous ligands of NK_2 binding sites which derived from some SP precursors, are present and coreleased from the same neurons in the brain (Diez-Guerra *et al*. 1988; Guard and Watson 1991). As for SP and NK_1 binding sites (Shults *et al*. 1984; Liu *et al*. 1994), there is no clear relationship between the regional distributions of NKA, NPK and NPγ (Arai and Emson 1986; Takeda *et al*. 1990) and of NK_2 binding sites. For instance, in the dorsal hippocampus which possesses the higher amount of NK_2 binding sites in the brain, the few NKA- or NPK-containing fibres present are not located in NK_2 labeled areas (Valentino *et al*. 1986; Shults *et al*. 1987). In contrast, a large amount of NKA is found in striatonigral neurons (Gerfen and Young 1988), but the striatum is practically devoid of NK_2 binding sites. This is surprising since NK_2 receptor-mediated responses induced by low concentrations of selective agonists and blocked by NK_2 antagonists, were demonstrated in this structure both *in vivo* and *in vitro* (Tremblay *et al*. 1992; Steinberg *et al*. 1998b). A rapid desensitization of NK_2 receptors occurring during the killing of the animals and the membrane preparation could eventually be responsible for this discrepancy, since partial receptor site occupancy is sufficient to desensitize cellular response to a second agonist application (Vollmer *et al*. 1999).

The occurrence of NK_2 binding sites in thalamic nuclei such as the posterior nuclear group (Po), the ventromedial (VM) or the ventral posterolateral (VPL) nuclei is of particular interest since these thalamic areas are known to contain nerve terminals of the reticulothalamic and spinothalamic neurons which convey nociceptive messages (Villanueva and Nathan 2000). In addition, electrophysiological responses evoked by cutaneous nociceptive thermal stimulation in the posterior thalamic nuclear group (Po) were shown to be blocked by the intravenous administration of the NK_2 antagonist SR 48968 (Santucci *et al*. 1993). Modifications in hippocampal levels of NKA and NPK have been demonstrated after repeated sensory stimulation, physical exercise, electroconvulsive treatment, kainic acid seizure or cerebral ischaemia (Sperk *et al*. 1990; Bucinskaite *et al*. 1996, 1998; Mathe 1999). In addition, self-sustaining status epilepticus in response to stimulation of the perforant path has been shown to induce a rapid and dramatic increase in the expression of preprotachykinin A mRNA in CA1 and CA3 pyramidal cell layers and damage (Liu *et al*. 1999b). Furthermore, a marked reduction in the duration and severity of seizures, as well as a prevention of both necrosis and apoptosis of CA1 and CA3 neurons induced by either kainate or pentylenetetrazole, have been observed in mice lacking the preprotachykinin A gene (Liu *et al*. 1999a). These observations, and our demonstration of NK_2 binding sites in hippocampal CA1 and CA3 areas suggest a critical role of messages mediated through NK_2 receptors in hippocampal excitability, seizure and vulnerability.

Comment should also be made on the limitations of this study. First, present preliminary autoradiographic data should be extended to further confirm the regional distribution of NK_2 binding sites and to obtain a more precise mapping of these binding sites for the interpretation of electrophysiological, biochemical or behavioural data. As well established, the affinity of the G protein-coupled receptor depends on the degree of coupling. The overall population of NK_2 receptors may thus not be labeled under our conditions. Using a labeled antagonist instead of an agonist should help to clarify this issue. Although similar

pharmacological properties of central and peripheral NK_2 binding sites were demonstrated in our study, unfortunately, convenient biological assays in the CNS which are required to confirm that central NK_2 binding sites do, in fact, correspond to functional receptors are presently lacking. In spite of these limitations, one of the most interesting aspects of our study is the demonstration that, in the brain, NKA, NPK and NPγ, which in most cases are colocated with SP, share the capacity to bind with a high affinity to NK_2 binding sites in addition to their high-affinity binding to NK_1 subtypes of binding sites (Beaujouan *et al*. 2000). These results explain why the central effects of NKA are blocked by either NK_2 or NK_1 antagonists, depending on the area or the biological response investigated. They also indicate that identification with appropriate antagonists of the implicated tachykinin receptor(s) should be made in further studies on the central effects of NKA, NPK or NPγ.

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