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Autoradiographic Localization and Characterization of Tachykinin Receptor Binding Sites in the Rat Brain and Peripheral Tissues

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Quantitative receptor autoradiography using several radiolabeled tachykinins was used to localize and characterize tachykinin peptide receptor binding sites in rat CNS and peripheral tissues. Autoradiographic localization and displacement experiments using several radiolabeled tachykinins indicate that in the rat there are at least 3 distinct tachykinin receptor binding sites. One of these is present in both the CNS and peripheral tissues, one is present only in the CNS, and one is present only in peripheral tissues.

The first tachykinin receptor binding site, which is detectable in both the CNS and peripheral tissues, appears to prefer substance P (SP) as an endogenous ligand. Areas expressing high concentrations of this binding site include the medial septum, superior colliculus, inferior olive, inner plexiform layer of the retina, external muscle of the bladder, and the muscularis externa of the esophagus.

The second type of tachykinin receptor binding site, which is detectable only in the CNS appears to prefer either neuromedin K (NK) and/or substance K (SK) as the endogenous ligand. This receptor binding site is labeled by Bolton-Hunter conjugates of NK, SK, eledoisin, or kassinin and is found in high concentrations in laminae 4 and 5 of the cerebral cortex, the ventral tegmental area, laminae 1 and 2 of the spinal cord, and the inner plexiform layer of the retina.

The third type of tachykinin receptor binding site is detectable only in peripheral tissues and appears to prefer SK as the endogenous ligand. This receptor binding site is labeled by SK, eledoisin, or kassinin radioligands and tissues that express high concentrations include the muscularis mucosae of the esophagus, the circular muscle of the colon, and the external muscle of the bladder.

These data suggest that SP receptors are expressed in the brain and peripheral tissues, NK receptors are expressed in the CNS, and SK receptors are expressed in peripheral tissue. These data fit well with radioimmunoassay data that suggest that, whereas in the CNS SP, SK *and* NK are present in high concentrations, in peripheral tissues *only* SP and SK are present in detectable concentrations. The present classification of tachykinin receptors places a lower limit on the number of mammalian tachykinin receptor types and provides a functional/morphological framework for exploring the diverse actions of tachykinin peptides in both the CNS and peripheral tissues.

The mammalian tachykinin family is currently known to be composed of 3 neuropeptides: substance P (SP), substance K (SK; neurokinin A, alpha-neurokinin, neuromedin L), and neuromedin K (NK; neurokinin B, beta-neurokinin). Since tachykinins are a family of peptides with the common carboxyl-terminal amino acid sequence -Phe-X-Gly-Leu-Met-NH_2 (Espamer, 1981; Maggio, 1988) (Table 1), it is important to know whether all of the tachykinins interact with a single receptor or whether each tachykinin utilizes its own specific receptor binding site. Previous studies are equivocal on this point as some studies suggest that the known mammalian tachykinins interact with a single tachykinin receptor binding site in peripheral tissues (Souquet et al., 1985), while other studies suggest that there are at least 2 distinct tachykinin receptor binding sites in peripheral tissues (Burcher et al., 1984, 1986; Mantyh et al., 1984d, 1987, 1988a, b; Buck and Burcher, 1985; Maggio et al., 1985).

Demonstration that pharmacologically relevant receptor binding sites are present on specific cell types is critical for assessing tachykinin action in the brain and peripheral tissues since it is believed that all tachykinin actions described to date are receptor-mediated (Pernow, 1983; Bartho and Holzer, 1985; Maggio, 1988). Demonstration of tachykinin receptor binding sites is also important since it is hypothesized that the presence of a particular neurotransmitter in a nerve does not necessarily imply that that particular neurotransmitter has a functional action on the innervated cell type (Dale, 1935; Eccles, 1957). While many neurons are known to contain multiple neurotransmitters, it has not been demonstrated that neurons are able to ship particular neurotransmitters only to those branches that innervate cells with postsynaptic receptors for that transmitter. This point is particularly germane to the tachykinins since the density of SP innervation and the density of SP binding sites do not correlate across CNS regions (Mantyh et al., 1984b, c, e; Schults et al., 1984). Furthermore, cloning of the bovine (Nawa et al., 1983, 1984), human (Harmar et al., 1986; Bonner et al., 1987), and rat (Bonner et al., 1987; Krause et al., 1987) cDNAs has revealed 4 tachykinin precursor proteins encoded by 2 genes. The first gene encodes for SP and SK and gives rise to 3 precursors. Two of these precursors, beta- and gamma-preprotachykinin A (or I), contain one copy each of SP and SK, while

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Table 1. Structure of tachykinins

Tachykinin	Structure
Substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Substance K	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neuromedin K	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Eledoisin	pGlu-Pro-Ser-Lys-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Kassinin	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
Common tachykinin sequence	-Phe-X-Gly-Leu-Met-NH_2

Sequence homologies are indicated by underlining. Mammalian tachykinins begin with a capital letter. Neurokinin-α, neurokinin A, and neuromedin L are alternative names for substance K. Neurokinin-β, neurokinin B are alternative names for neuromedin K. H- denotes a free amino terminus, -NH_2 a carboxyl terminal carboxyamide, and pGlu a pyroglutamic acid moiety.

the other precursor, alpha-preprotachykinin A, contains a single copy of SP. The second gene encodes preprotachykinin B (or II), a precursor for NK only; the mRNA for NK has been reported to be present in the bovine brain and small and large intestine (Kotani et al., 1986). Radioimmunoassay data is in general agreement with the molecular genetics data in that SP and SK coexist in most areas of the brain and periphery and appear to be co-released in all systems examined to date (Kanazawa et al., 1984; Maggio and Hunter, 1984; Deacon et al., 1987). NK, on the other hand, appears to be expressed in a different pattern than SP and SK in the brain and has not been detected in peripheral tissues and sensory ganglia in the rat (Minamino et al., 1984; Ogawa et al., 1985; Deacon et al., 1987).

In the present report we have used quantitative receptor autoradiography to explore the characteristics and distribution of tachykinin receptors in the rat brain and peripheral tissues. To accomplish this we have tested 19 different radioligands on rat brain and peripheral tissues and from these 19 radioligands report in detail on 5 that consistently gave good resolution with high specific/nonspecific binding ratios. Thus, we have used radiolabeled analogs of the 3 mammalian tachykinins (substance P, substance K, and neuromedin K) and 2 nonmammalian tachykinins (kassinin and eledoisin) that have been used extensively in previous physiological and pharmacological experiments to demonstrate the existence of multiple tachykinin receptors.

Materials and Methods

In the present study, several tachykinin radioligands were tested for suitability in autoradiographic studies. These radioligands, their abbreviations, and origin are listed in Table 2 below. Complete peptide sequences are given in Table 1. The radioligands synthesized in our laboratory were prepared from commercially available peptide precursors and reagents by conventional radioiodination techniques (Hunter and Greenwood, 1962; Bolton and Hunter, 1973; Cascieri and Liang, 1984). Following synthesis, the radioiodinated peptides were purified by reverse-phase HPLC to essentially quantitative specific activity (2000 Ci/mmol), diluted to less than 10^8 dpm/ml, and stored at −20°C in solutions containing 0.2% 2-mercaptoethanol as antioxidant. Under these conditions, the monoiodinated radioligands were stable for at least 3 months, and the diiodinated ligands for at least 1 month. The radioligand stock solutions were diluted to a working concentration of 100 pM shortly before incubation with tissue sections. Except as noted, all peptides were from Bachem, all radioisotopes from Amersham, and all chemicals and biochemicals from Sigma.

Rats were sacrificed by decapitation and tissues rapidly dissected out. Tissues were then blocked, placed on a brass microtome chuck, and frozen on dry ice. The tissues were then serially sectioned (30 μm), thaw-mounted onto gelatin-coated microscope slides, and stored at −20°C over desiccant for up to 3 months.

For autoradiography with the BHSP (radioligand 3 in Table 1), the slide-mounted tissue sections were brought to room temperature and placed in a preincubation medium (50 mM Tris-HCl, pH 7.4, containing 0.005%, vol/vol, polyethylenimine) at 19°C for 10 min before incubation with radioligand. The sections were then incubated at 19°C for 1 hr in a solution of 100 pM BHSP in 50 mM Tris-HCl, pH 7.4, containing $MnCl_2$ (3 mM), BSA (200 mg/liter), chymostatin (2 mg/liter), leupeptin (4 mg/liter), and bacitracin (40 mg/liter). To conserve radioligand, the incubation was accomplished by placing the slides on a horizontal surface and covering the sections with the incubation medium. HPLC analysis confirmed that under these conditions, no significant degradation or oxidation of radioligand is observed during the course of the experiments. To estimate nonspecific binding, paired serial sections were incubated as described except that 1 μM SP was present in the incubation solution. Following incubation with radioligand, slides were rinsed twice in 50 mM Tris-HCl, pH 7.4 (4°C, 2 min each), then 4 times in distilled water (4°C, 5 sec each), and then quickly dried in the cold room using a stream of cold air. After 3 hr further drying at 4°C, sections were stored over desiccant at room temperature overnight before autoradiography. The same procedure was used for the I_2-BHSP (radioligand 4); the doubly iodinated ligand consistently gave a lower specific/nonspecific binding ratio than the mono-iodo derivative. The tritiated ligands ^{3}H-SP and ^{3}H-PrSP (radioligands 1 and 2), being of significantly lower specific activity than the iodinated ligands, were used at a concentration of 2 nM; otherwise, the procedure was as described above. Controls for chemographic artifacts were performed as above except that radioligand was omitted from the incubation medium.

For the SK and NK radioligands, the same protocol was employed except that polyethylenimine was omitted from the preincubation, the length of the incubation increased to 2 hr and the pH of the incubation medium was increased to 8.0 for the SK radioligands and 8.5 for the NK radioligands; nonspecific binding was estimated using 1 μM SK or NK. The Bolton-Hunter conjugates of kassinin and eledoisin (ligands 16 and 17 in Table 2) were studied under the conditions used for SK radioligands. the 5 radioligands (numbered 5, 9, 10, 18, 19 in Table 2) which failed to give specific binding under the above protocols were further studied under a variety of other conditions (varying incubation and wash times, pH, salt, and temperature) without success.

For quantitative autoradiographic analysis of tachykinin binding sites, the slide-mounted tissue sections were placed in apposition to tritium-sensitive Ultrofilm (LKB). Radioiodinated brain mash or standards (see below) exposed simultaneously provided the basis for quantitation. After 1–4 weeks exposure, the film was developed in Kodak D-19 developer, fixed, and washed. In those cases where a higher degree of histological resolution was desirable, the same slide-mounted sections were processed for standard emulsion-dipped autoradiography following fixation by formaldehyde vapor (Herkenham and Pert, 1982). After the emulsion-dipped autoradiograms were developed, the sections were placed in Carnoy's fixative for 3 hr, stained with hematoxylin and eosin or cresyl violet, and mounted with Histoclad. Dark- and bright-field photomicrographs were then taken of the silver grains and counterstained sections, respectively. Using this approach, 3 complementary images were generated: the film autoradiogram, which was analyzed for quantitative densitometry; the emulsion autoradiogram, which provided detailed histological resolution of binding sites; and the counterstained section, which allowed identification of the cell types expressing the binding sites.

Table 2. Radioligands used in the present study

Abbreviation	Radioligand	Notes
1. ^{3}H-SP	Substance P, [prolyl^{2}-3,4(n)-^{3}H]	*a*
2. ^{3}H-PrSP	Substance P, [Na-^{3}H-propionyl]	*b*
3. BHSP	Substance P, [Na-^{125}-monoiodo-Bolton-Hunter]	*a–c*
4. I_2-BHSP	Substance P, [Na-($^{125}I_2$)-diiodo-Bolton-Hunter]	*c*
Substance K radioligands		
5. ^{3}H-PrSK	Substance K, [Na-^{3}H-propionyl]	*b*
6. BHSK	Substance K, [Na-^{125}I-monoiodo-Bolton-Hunter]	*c*
7. I_2-BHSK	Substance K, [Na-($^{125}I_2$)-diiodo-Bolton-Hunter]	*c*
8. ISK	Substance K, [2-^{125}I-iodohistidyl^{1}]	*b, c*
9. BHSK (4–10)	Substance K$^{4-10}$ heptapeptide, [Na-^{125}I-monoiodo-Bolton-Hunter]	*c*
10. IY^{0}-SK	Substance K, [Na-(m-^{125}I-iodotyrosyl)]	*c*
Neuromedin K radioligands		
11. ^{3}H-PrNK	Neuromedin K, [Na-^{3}H-propionyl]	*b*
12. BHNK	Neuromedin K, [Na-^{125}I-monoiodo-Bolton-Hunter]	*c*
13. I_2-BHNK	Neuromedin K, [Na-($^{125}I_2$)-diiodo-Bolton-Hunter]	*c*
14. INK	Neuromedin K, [2-^{125}I-iodohistidyl^{5}]	*c*
15. IY^{0}-NK	Neuromedin K, [Na-(*m*-^{125}I-iodotyrosyl)]	*c*
Other radioligands		
16. BHK	Kassinin, [Na-^{125}I-monoiodo-Bolton-Hunter]	*c*
17. BHE	Eledoisin, [Ne-^{125}I-monoiodo-Bolton-Hunter]	*c*
18. BH-senktide	[Asp^{5,6},MePhe^{8}]-substance P$^{5-11}$, [Na-^{125}I-monoiodo-Bolton-Hunter]	*c*
19. BHNPK	Neuropeptide K, [Ne-^{125}I-monoiodo-Bolton-Hunter]	*c*

a New England Nuclear.
b Amersham.
c Synthesized in our laboratory.

To quantitate the density of radiolabeled tachykinin binding sites, microdensitometry (Rainbow et al., 1984) was performed on the film autoradiograms. The developed film was projected at 20× on a white horizontal surface and the densities of areas of the projected image measured with a photocell (Sharp BS-5900A silicon blue photodiode) connected to a digital voltmeter (Radioshack). The resolution of this device corresponds to region about 20 μm in diameter on the projected sections. Correction for the film's nonlinear response to radioactivity was performed using film exposed to series of radioactive standards and an automatic curve-fitting program (Texas Instruments).

Results

Radioligands tested

Radioligands that preferentially bind to SP receptors. Of the 19 radioligands tested, 4 (numbers 1–4) preferentially bind to SP receptors. ^{3}H-SP has proved to be an extremely good ligand when used with polyethylenimine (PEI) and purified by HPLC immediately before use, since it gives very high (95/5) specific/nonspecific binding ratios. The disadvantage of this ligand is that, like all ^{3}H-ligands used for autoradiographic analyses, gray/white quenching differences must be taken into account (Herkenham and Sokoloff, 1984). However, if these correction factors are incorporated and tritiated microscales (Amersham) are employed, reliable quantitative autoradiography can be performed. The ^{125}I-SP ligands we have tested appear to be very good for labeling SP receptors since they produce high (95/5, for mono-iodo Bolton-Hunter SP) specific/nonspecific binding ratios and do not have the differential quenching problems of ^{3}H-ligands, although their relatively short half-lives make it necessary to generate new microscales for each experiment to properly quantify the results. All 4 of the SP radioligands appear to label a single class of SP receptor which shows little affinity for SK or NK; the IC_{50} of SK and NK in displacing these radioligands is 3 orders higher than that for SP.

Substance K radioligands. Three SK radioligands, mono- and di-iodinated Bolton-Hunter conjugates and the "internally labeled" iodohistidyl derivative (numbers 6, 7, 8 respectively, in Table 2) label binding sites in the brain and peripheral tissues of the rat. Each gives high specific/nonspecific binding ratios, and at least in the periphery appears to preferentially label SK receptors. BHSK (number 6 in Table 2) consistently gave the highest specific/nonspecific binding ratios (95/5) of the ligands tested. Three other SK radioligands, the propionylated and tyrosylated derivatives (numbers 5 and 10 in Table 2) and the Bolton-Hunter conjugate of the carboxyl-terminal heptapeptide (number 9 in Table 2), did not exhibit specific binding in either central or peripheral tissues under the protocols described above nor under a variety of other conditions tested. Bolton-Hunter conjugates of kassinin and eledoisin (numbers 16 and 17 in Table 2) also appear to label SK receptors.

Neuromedin K radioligands. Five radioligands have been synthesized to explore the distribution of NK receptors. All appear to bind to the same site in the CNS; however, the internally labeled iodohistidyl NK (number 14 in Table 2) appears to give the highest specific/nonspecific binding ratios of 80/20). BHK and BHE also appear to label NK receptors in the rat brain.

Other tachykinin radioligands. The Bolton-Hunter conjugates of kassinin and eledoisin (ligands 16 and 17 in Table 2) label tachykinin binding sites in both central and peripheral tissues, giving patterns that appear very similar to those of BHSK in the same tissues.

We were unable to demonstrate any specific binding of the Bolton-Hunter derivative of senktide (ligand 18 in Table 2), a

Table 3. Distribution of tachykinin receptor binding sites

Tissue	SP	NK	SK	E	K
Central nervous system					
Olfactory bulb					
External plexiform layer	++++	+	+	+	+
Internal granule layer	+	+	+	+	+
Cerebral Cortex					
Anterior cingulate					
I	+	+	+	+	+
II–III	+	–	–	–	–
IV–V	–	++++	++++	++++	++++
Frontal-parietal					
I	+	+	+	+	+
II–III	+	–	–	–	–
IV–V	–	++++	++++	++++	++++
Temporal					
I	+	+	+	+	+
II–III	+	–	–	–	–
IV–V	–	++++	++++	++++	++++
Entorhinal					
I–III	+++	+	+	+	+
IV-V	+	+++	+++	+++	+++
Septum					
Medial	++	–	–	–	–
Lateral	++++	–	–	–	–
Nucleus accumbens	+++	–	–	–	–
Caudate-putamen	+, +++	–	–	–	–
Amygdalohippocampal area	++++				
Amygdala					
Basal lateral nucleus	++	+++	+++	+++	+++
Central nucleus	++	–	–	–	–
Lateral nucleus	+	–	–	–	–
Medial nucleus	+	–	–	–	–
Hippocampus					
CA3	++	+	+	+	+
Dentate gyrus	++	+	+	+	+
Subiculum	++++	–	–	–	–
Dorsal thalamus					
Anterior nuclei	++	–	–	–	–
Lateral geniculate nucleus	–	–	–	–	–
Medial geniculate nucleus	+	–	–	–	–
Ventral posterior medial nucleus (parvocellularis)	++	+	+	+	+
Ventral posterior lateral nucleus	–	–	–	–	–
Hypothalamus					
Anterior nucleus	+++	–	–	–	–
Dorsomedial nucleus	+	–	–	–	–
Lateral nucleus	+	–	–	–	–
Paraventricular nucleus	++	++++	++++	++++	++++
Supraoptic	–	++++	++++	++++	++++
Ventral Thalamus					
Ventral lateral geniculate nucleus	+	–	–	–	–
Epithalamus					
Habenular nucleus					
Medial	++	+	+	+	+
Lateral	+	–	–	–	–
Paraventricular thalamic nucleus	+++	–	–	–	–

Table 3. Continued

Tissue	SP	NK	SK	E	K
Mesencephalon					
Interpeduncular nucleus	++	++	++	++	++
Periaqueductal grey					
Dorsal	++	+	+	+	+
Lateral	++	–	+	+	+
Medial	++	+	+	+	+
Substantia nigra					
Compacta	+	+	+	+	+
Reticulata	–	–	–	–	–
Superior colliculus					
Superficial layer	++++	+	+	+	+
Intermediate layer	+++	–	–	–	–
Deep layer	++	–	–	–	–
Third nucleus	+	–	–	–	–
Ventral tegmental area	–	+	+	+	+
Pons					
Cerebellum granule layer					
(9 and 10)	++	–	–	–	–
Dorsal raphe	+++	–	–	–	–
Fourth nucleus	+	–	–	–	–
Inferior colliculus	+	–	–	–	–
Locus coeruleus	++++	+	+	+	+
Parabrachial nucleus					
Medial	++	–	–	–	–
Lateral	++++	–	–	–	–
Medulla					
Ambiguous	++	–	–	–	–
Dorsal motor nucleus of the					
vagus	++++	–	–	–	–
Inferior olive					
Medial	++	–	–	–	–
Lateral	++	–	–	–	–
Raphe nuclei					
Magnus	+	–	–	–	–
Pallidus	+	–	–	–	–
Obscuris	+	–	–	–	–
Solitary nucleus					
Medial	+++	++++	++++	++++	++++
Lateral	++	++++	++++	++++	++++
Trigeminal nucleus					
Oralis	+	–	–	–	–
Interpolaris	+	–	–	–	–
Caudalis					
I–II	++	+++	+++	+++	+++
V	+	–	–	–	–
Spinal cord					
Cervical					
DRG	–	–	–	–	–
I–II	++	++++	++++	++++	++++
III–IV	–	–	–	–	–
V	+	–	–	–	–
VI–IX	++	–	–	–	–
Area X	+++	–	–	–	–
Thoracic					
DRG	–	–	–	–	–
I–II	++	++++	++++	++++	++++
III–IV	–	–	–	–	–
V	+	–	–	–	–

Table 3. Continued

Tissue	SP	NK	SK	E	K
VI–IX	++	–	–	–	–
Area X	++	–	–	–	–
IML	++++	–	–	–	–
Lumbar					
DRG	–	–	–	–	–
I–II	++	++++	++++	++++	++++
III–IV	–	–	–	–	–
V	+	–	–	–	–
VI–IX	++	–	–	–	–
Area X	+++	–	–	–	–
Sacral					
DRG	–	–	–	–	–
I–II	++	++++	++++	++++	++++
III–IV	–	–	–	–	–
V	+	–	–	–	–
VI–IX	++	–	–	–	–
Area X	+++	–	–	–	–
Sacral preganglionic	++++	–	–	–	–
Onuf's	++++	–	–	–	–
Retina (inner plexiform layer)	+++	++	++	++	++
Peripheral tissues					
Esophagus					
Muscularis mucosae	++	–	++	++	++
Muscularis externa	+	–	–	–	–
Colon					
Circular muscle	++++	–	+++	+++	+++
Longitudinal muscle	+++	–	++	+	+
Bladder					
Muscularis externa	++++	–	++++	++++	++++

Serially adjacent frozen brain sections (30 μm) were labeled as described in the text with ^{125}I-BHSP, ^{125}I-BHNK, ^{125}I-BHSK, ^{125}I-BHE, or ^{125}I-BHK and opposed to LKB Ultrofilm for 10 d. Densitometric readings for each region were taken as described from at least 5 different animals, averaged and corrected for the nonlinearity of the response of the film using autoradiographic standards (Amersham). Values are expressed as a percentage of maximal binding from each ligand where (–) is undetectable, (+) 0.1–25.0%, (++) 25.1–50.0%, (+++) 50.1–75.0%, (++++) 75.1–100.0%. The area with the maximal density of BHSP binding sites is the amygdalohippocampal area, whereas for BHNK, BHSK, BHE, and BHK, it is the supraoptic hypothalamic nucleus.

synthetic tachykinin derivative reported (Laufer et al., 1986) to specifically label NK binding sites, in any area of the rat brain or periphery examined. With a mixture of 4 different Bolton-Hunter derivatives of neuropeptide K (ligand 19 in Table 2), we were also unable to detect any specific binding in any of the central or peripheral tissues examined. It should be emphasized that these radioligands were tried without success under a variety of binding conditions, and the tissues that showed no specific binding of these 2 ligands were shown in parallel experiments done the same day to exhibit specific binding for the other tachykinin radioligands.

Competitive inhibition of ^{125}I-Bolton-Hunter SP, SK, and NK binding by SP, SK, and NK

The first type of tachykinin binding site is detectable in both brain and peripheral tissues (Table 3) and is here referred to as the SP binding site since SP is about 3 orders of magnitude more potent than either SK or NK in displacing BHSP from this binding site. The SP binding site has a K_d of approximately 1.0 nM (Mantyh et al., 1984c) and the SP receptor expressed by cells in the gastrointestinal (GI) tract (Burcher et al., 1986) appears to have a similar pharmacology to that of the SP receptor in brain (Torrens et al., 1983).

The second binding site is found only in the CNS (Table 3) and is here referred to as the NK binding site. Both NK and

Table 4. Tachykinin receptor nomenclature

Investigator(s)		SP	SK	NK
Henry et al., 1987		NK-1	NK-2	NK-3
Henry et al., 1987		TK-1	TK-2	TK-3
Henry et al., 1987		SP-P	NK-A	NK-B
Laufer et al., 1985		SP-P	SP-E	SP-N
Regoli et al., 1985		NK-P	NK-A	NK-B
Piercey et al., 1985		SP_2	SP_1	SP_1
Buck et al., 1984a		SP-P	SP-K	SP-E
Lee et al., 1982	SP-P	SP-P	SP-E	SP-E
Melchiorri and Negri, 1984	TK-2	TK-1	TK-3	TK-3
Erspamer, 1981	PHYS	SP	KAS	ELE
Agonist	PHYS	SP	K	E

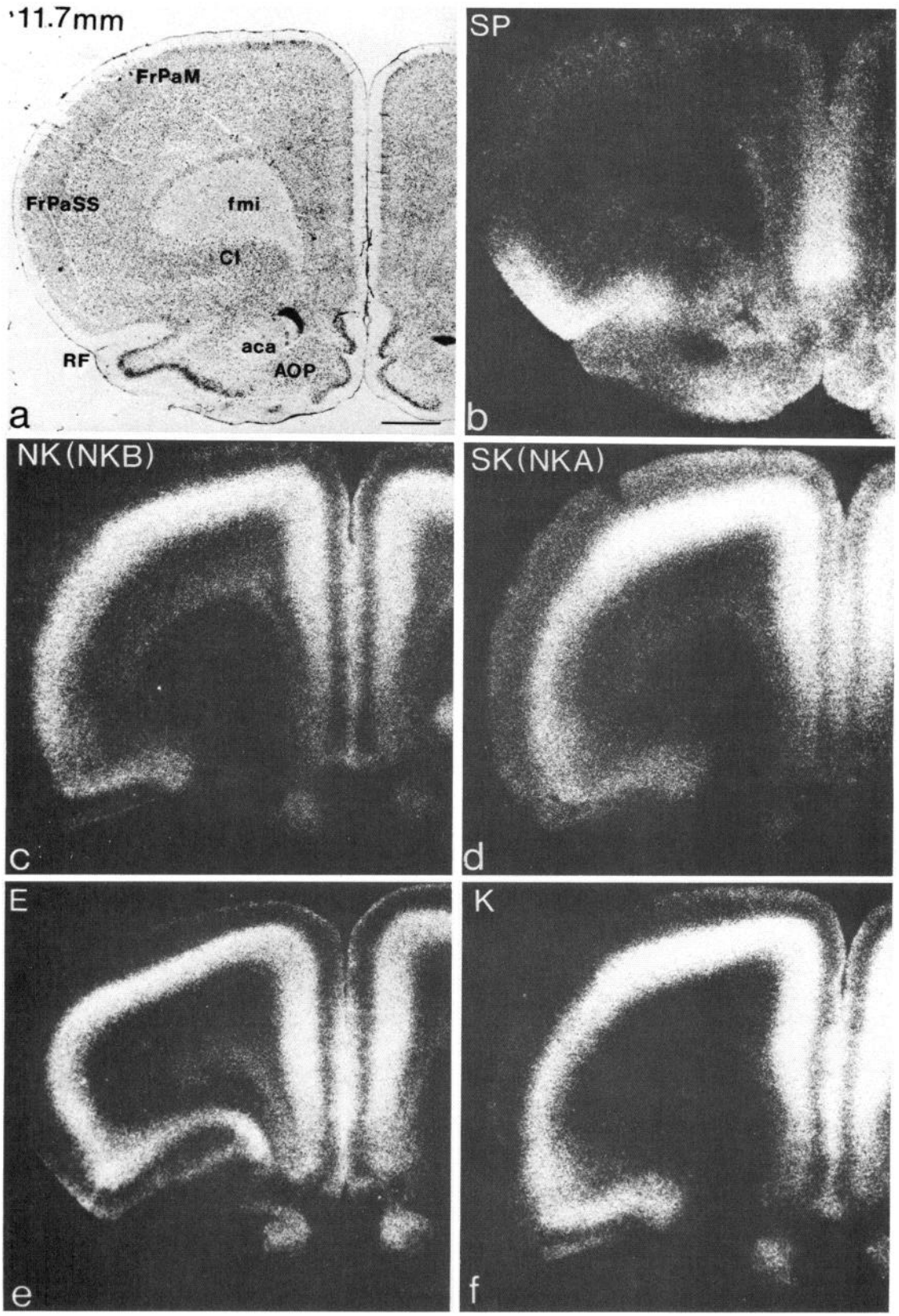

Figure 1. Autoradiographic localization of tachykinin receptor binding sites in coronal sections of the rat brain. In Figures 1–6, *a* is a light-field photomicrograph of the rat brain section that was stained with cresyl violet to aid in the orientation of the tissue section, whereas *b–f* are dark-field autoradiograms of adjacent sections that demonstrated the distribution of binding sites for the following Bolton-Hunter labeled tachykinins: *b,* substance P (SP); *c,* neuromedin K (NK); *d,* substance K (SK); *e,* eledoisin (E); and *f,* kassinin (K). For orientation purposes, we have given the distance each coronal section lies in front of the interaural line in upper left-hand corner of *a.* All abbreviations are from the atlas of Paxinos and Watson (1982). In the photomicrographs *b–f,* the light areas correspond to concentrations of binding sites. Unless otherwise noted, all white areas

SK are more potent than SP in displacing either NK or SK from these sites, but NK appears to be more potent than SK in displacing either BHNK or BHSK from rat brain sections. Thus, in laminae 4 and 5 of the rat cerebral cortex the K_i's for displacing BHNK are 0.1 nM for NK, 1.0 nM for SK, and 95 nM for SP (see Fig. 11*A*), whereas in this same tissue the K_i's for displacing BHSK are 0.1 nM for NK, 1.0 nM for SK, and 40 nM for SP (Fig. 11*B*).

The third type of receptor that is found only in peripheral tissues (Table 3) is here referred to as the SK binding site since both SP and NK appeared to be less potent than SK in displacing BHSK from its peripheral binding site and since no BHNK binding sites have been found in any peripheral tissue we examined. This binding site in the external circular muscle of the rat colon has a K_d of 2.0 nM and K_i's of 3000 nM for SP, 2.5 nM for SK, and 90 nM for NK (Fig. 11*C*). This site appears to be expressed only in the GI tract and has not been reported to be present in the brain.

To date, no single system of tachykinin receptor nomenclature has yet become conventional. We refer here to the observed binding sites as SP, SK, and NK without prejudice toward the various nomenclatures (Table 4) currently in use (see below).

Comparison of specific binding sites for ^{125}I-Bolton-Hunter SP, SK, NK, E, and K in the CNS

In general, the distribution of SP, NK, SK, K, and E binding sites in the brain is in close agreement with previous findings (Shults et al., 1982, 1984; Quirion et al., 1983; Quirion and Pilapil, 1984; Mantyh et al., 1984a–e, 1987; Rothman et al., 1984a, b; Beaujouan et al., 1986; Buck et al., 1986). In describing the distribution of binding sites we will therefore concentrate on areas where a clear difference between SP and NK binding could be seen. Probably the most prominent difference between SP and NK binding sites is in the cerebral cortex. As shown on Figures 1–5, there is a heavy concentration of NK binding sites in laminae 4 and 5 of the cerebral cortex, whereas SP binding sites are detectable and only in low concentrations in laminae 1 and 2 of the cerebral cortex. The one area of cortex that expressed a high concentration of SP binding sites was the striate and entorhinal cortex, a region of the cortex involved in gustatory and visceral integration.

Other areas showing a marked difference between SP and NK binding sites are the ventral tegmental area and superficial superior colliculus (Figs. 4, 5). Whereas there are only very low concentrations of SP binding sites in the VTA, there are moderate concentrations of NK binding sites in this region (Fig. 4). In the superficial layer of the superior colliculus, this situation is reversed. Here, one finds a very heavy concentration of SP binding sites but only a low concentration of NK binding sites.

There is a comparable situation in the dorsal raphe and median raphe. In the dorsal raphe there is a very heavy concentration of SP binding sites but only a low concentration of NK binding sites. This is reversed in the median pontine raphe; there is a moderate concentration of NK binding sites but few if any SP binding sites in this nucleus.

In the brain stem there are several areas where there is a marked difference in the expression of SP and NK binding sites (Fig. 6). SP binding sites are found in high concentrations over several motor nuclei (including 7, 10, 12, and ambiguous), whereas NK binding sites are undetectable in these motor nuclei. This is in contrast to sensory nuclei such as the nucleus of the solitary tract, where there are very high concentrations of both NK and SP binding sites. This distinction of NK binding sites being associated with sensory regions, whereas SP binding sites are associated with both motor and sensory regions also holds true for the spinal cord. Whereas SP binding sites are expressed by areas of the spinal cord involved in both sensory (laminae 1 and 2) and motor (laminae 6–9, Onuf's nucleus) function, NK binding sites are expressed only in laminae 1 and 2, which are known to be involved in sensory functions. In the retina specific binding sites for SP, SK, NK, E, and K are present over the inner plexiform layer (Fig. 7).

Comparison of specific binding sites for ^{125}I-Bolton-Hunter SP, SK, NK, E, and K in peripheral tissues

Unlike the brain, where BHNK, BHSK, BHE, and BHK labeled similar brain areas in peripheral tissues no BHNK sites could be detected, whereas BHSK, BHE, and BHK labeled similar structures. For this reason and because of the displacement studies (Fig. 11*C*), we refer to these peripheral binding sites as SK sites.

In the *esophagus,* SP binding sites are present in low to moderate concentrations over the muscularis externa and in low concentrations over the muscularis mucosae, whereas SK binding sites are found in high concentrations only in the muscularis mucosae (Fig. 8). In the *colon* (Fig. 9), SP and SK binding sites were expressed in high concentrations by the external circular muscle and in low concentrations by neurons in the myenteric plexus and longitudinal muscle. In the *bladder* (Fig. 10), both SP and SK binding sites were expressed in very high concentrations by the external muscle.

Variability of specific binding sites for ^{125}I-Bolton-Hunter SP, SK, NK, E, and K in the CNS and peripheral tissues

The distribution and concentration of tachykinin binding sites did not vary significantly from animal to animal or day to day with 2 notable exceptions. The first was binding in the striatum. In previous experiments we and others showed that a high concentration of SP binding sites was present in the striatum and that this appeared to be localized to neurons as kianic acid injection into the striatum greatly reduced the number of SP binding sites (Mantyh and Hunt, 1986). In rat R4, however, we did not observe a high concentration of binding sites in the striatum using either BHSP or ^{3}H-SP (New England Nuclear) run on different days. In 10 other rats, BHSP binding in the striatum was normal.

The second variable binding was in rat R7 (Fig. 3), which had normal SP binding (including striatum) but did demonstrate differences in BHNK, BHSK, BHE, and BHK binding in the hippocampus and medial habenula. We repeated the binding experiments on rat R7 using all the ligands on subsequent days with the same results. In 9 other rats, the patterns of BHNK, BHSK, BHE, and BHK were very similar in these areas and appeared similar to Figure 3*c*.

←

correspond to specific binding sites in that labeling was absent in adjacent sections when an excess (10^{-6} M) of cold ligand was added to the incubation medium. Here (Fig. 1), notice that the highest concentration of binding sites for SP is over the outer laminae of the insular cortex, whereas for NK, SK, E, and K the binding sites are densest over laminae 4 and 5. Scale bar, 1 mm.

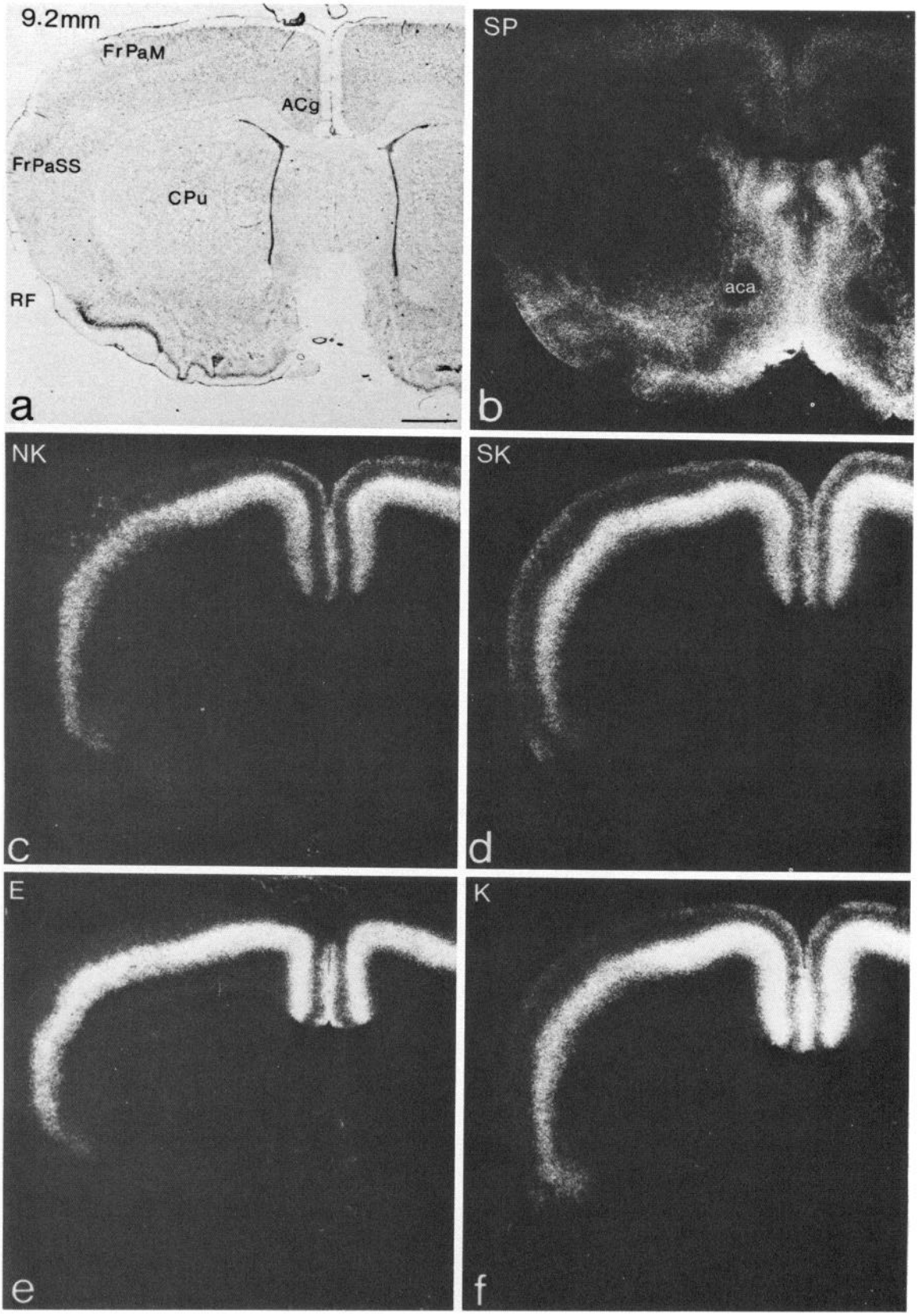

Figure 2. Autoradiographic localization of tachykinin binding sites in the cerebral cortex and striatum. Note the high concentration of NK, SK, E, and K binding sites in laminae 4 and 5 of the cerebral cortex, whereas SP binding sites are expressed in high concentrations by the nucleus accumbens. See Figure 1 for explanation. Scale bar, 1 mm.

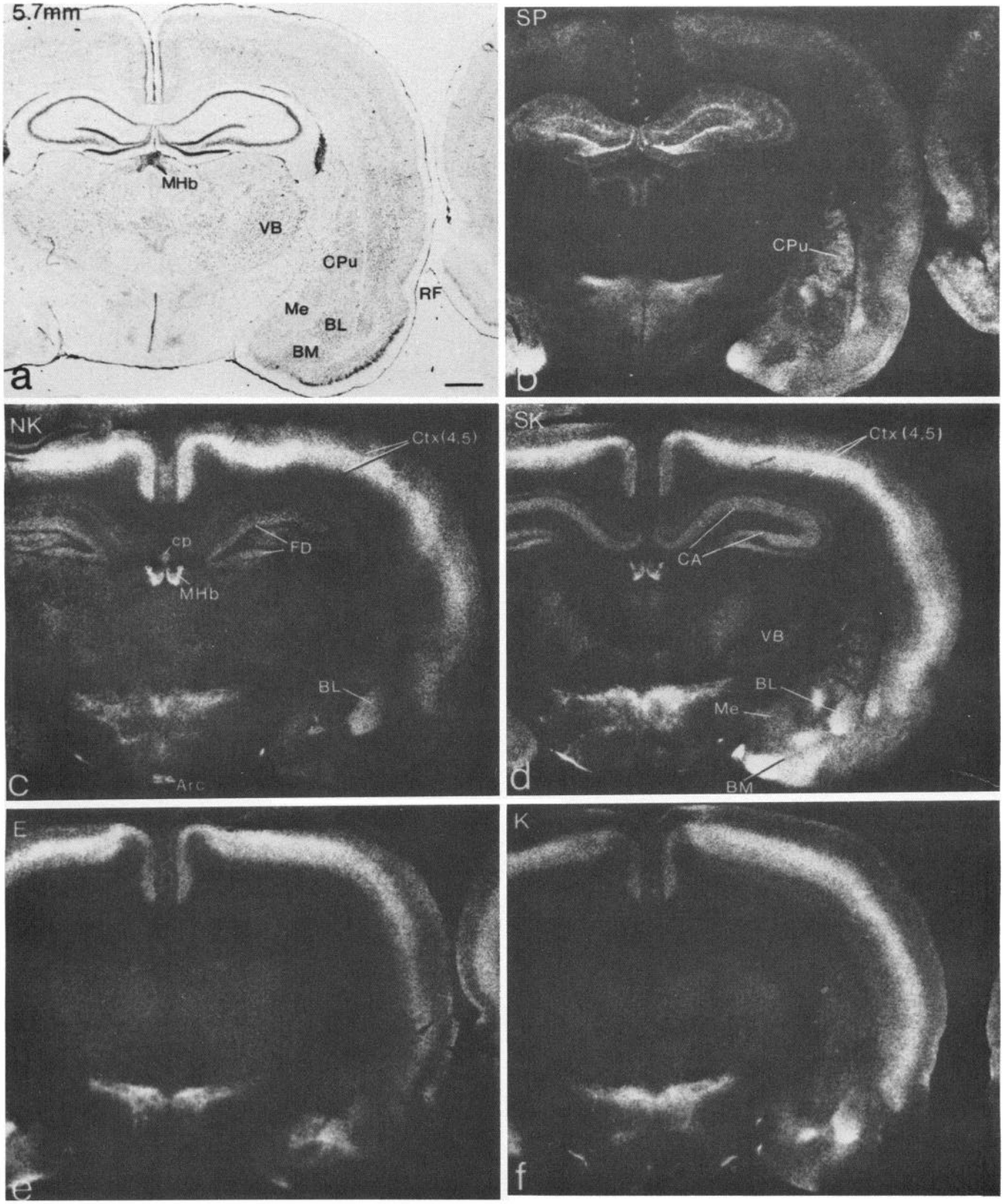

Figure 3. Autoradiographic localization of tachykinin binding sites in the cerebral cortex, hippocampus, hypothalamus, and amygdala in rat R7. The major differences to note are that, whereas NK, SK, E, and K are present in high concentrations in laminae 4 and 5 of the cerebral cortex, SP binding concentrations are present in low concentration in laminae 1 and 2. Note also that in *c–f* there are minor differences in the location and concentration of binding sites among these 4 ligands. For example, whereas BHNK and BHSK binding sites are expressed by the medial habenula, BHE and BHK sites are not seen in *e* and *f*. This was the only rat of the 10 we examined in which we observed these differences. In all other animals, SK, NK, E, and K binding sites were nearly identical and were similar to *c* or *d*. See Figure 1 for explanation. Scale bar, 1 mm.

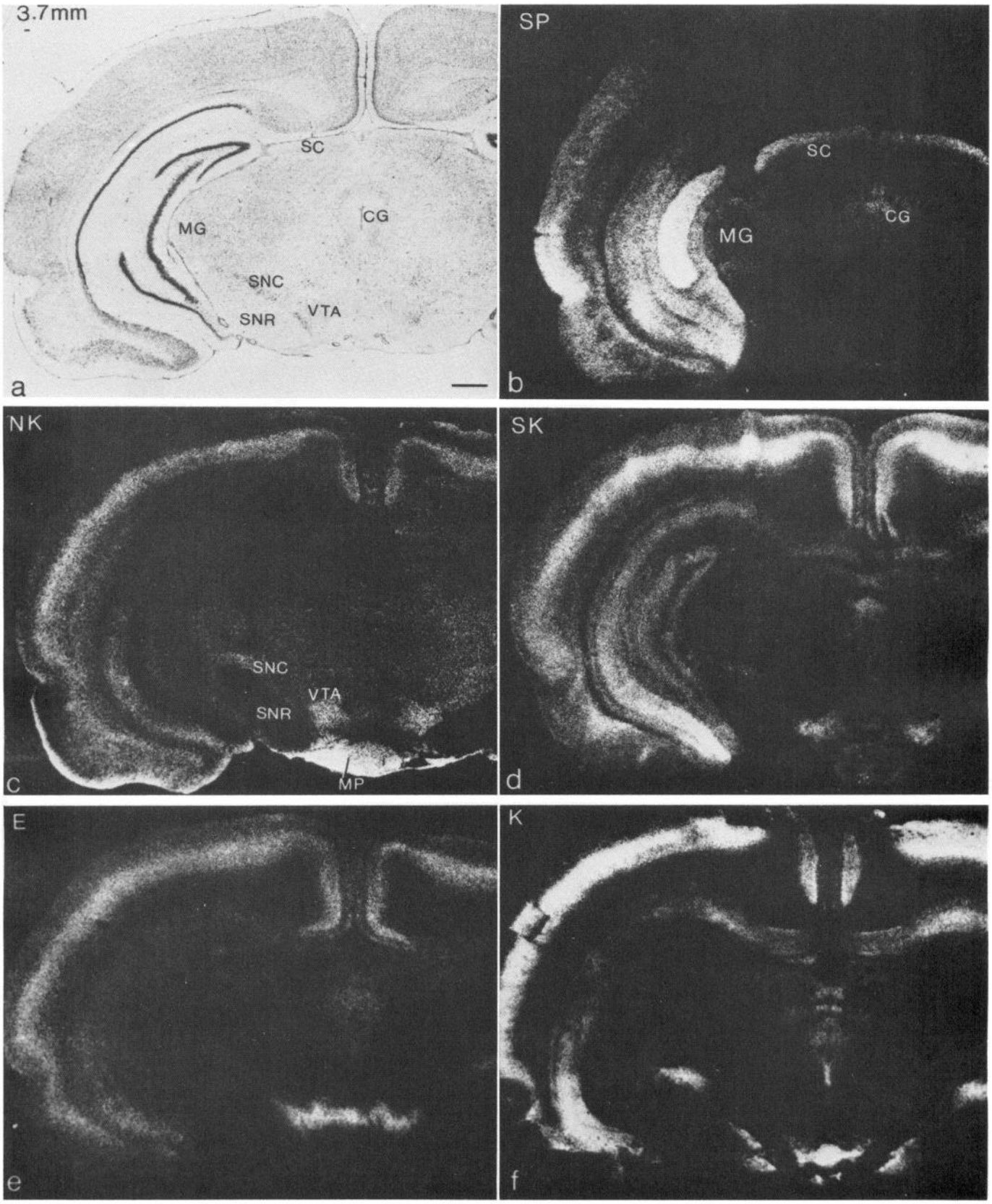

Figure 4. Autoradiographic localization of tachykinin binding sites in the cerebral cortex, hippocampus, and midbrain. Note the moderate concentration of NK, SK, E, and K binding sites in the ventral tegmental areas, whereas only a low concentration of SP binding sites is present in this area. See Figure 1 for explanation. Scale bar, 1 mm.

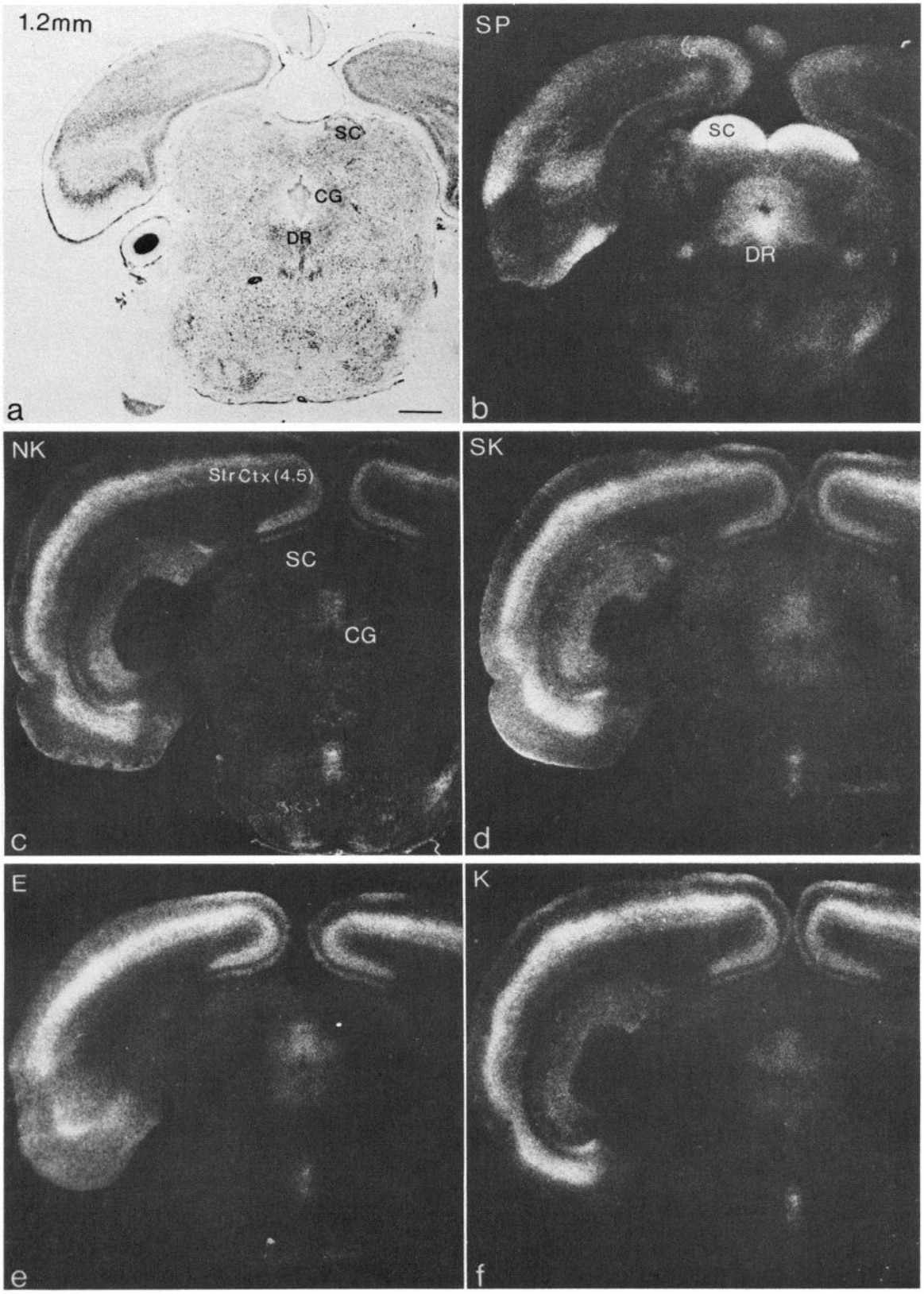

Figure 5. Autoradiographic localization of tachykinin binding sites in the striate cortex and midbrain. Note the differences in the distribution of SP versus NK, SK, E, and K binding sites in the striate cortex. Also note that, whereas SP binding sites are expressed in high concentrations in the superficial layers of the superior colliculus and dorsal raphe, NK, SK, E, and K binding sites are expressed in low concentrations in these areas. See Figure 1 for explanation. Scale bar, 1 mm.

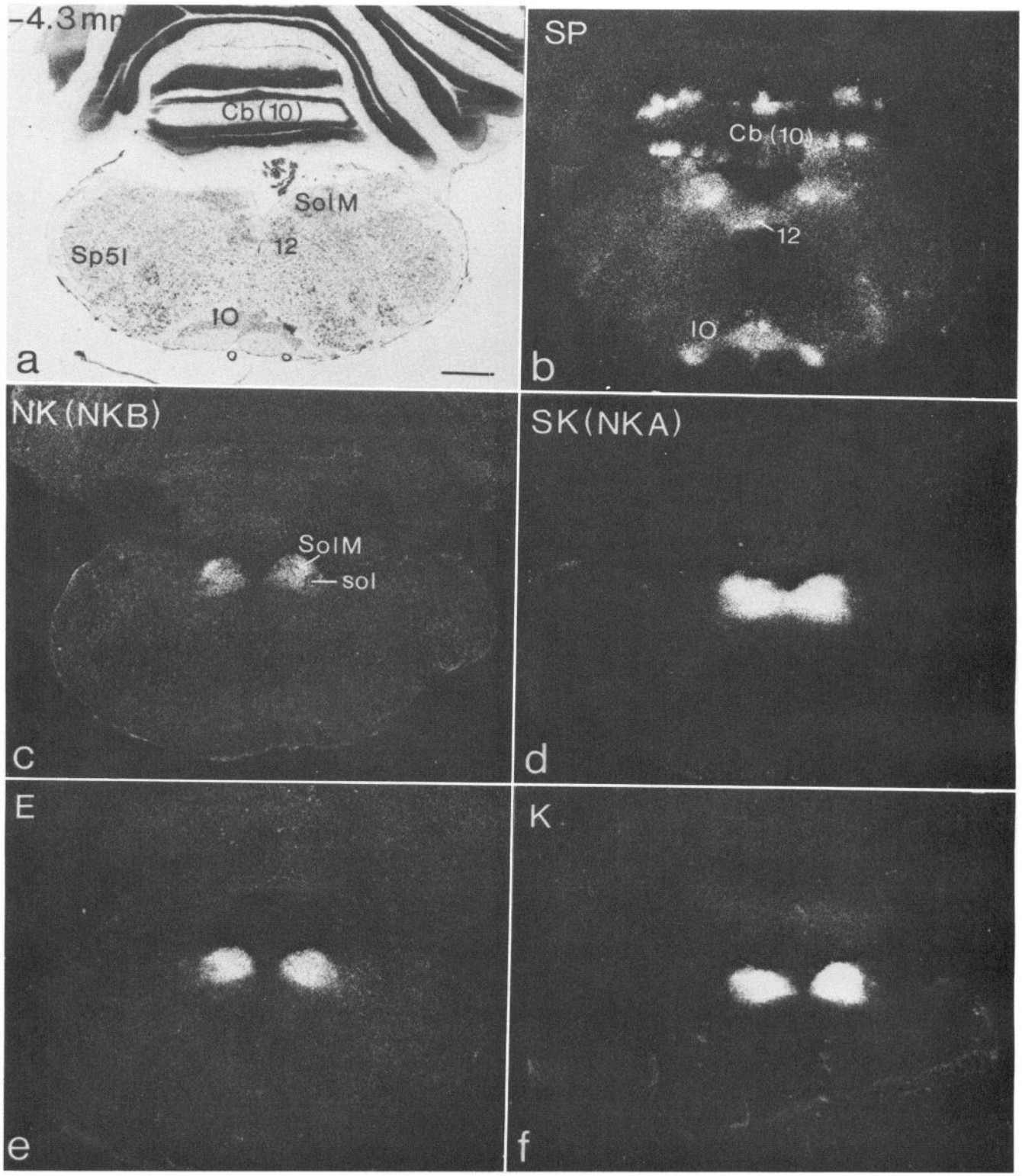

Figure 6. Autoradiographic localization of tachykinin binding sites in the medulla and cerebellum. Note that, whereas SP binding sites are expressed by lobule 10 in the cerebellum, the hypoglossal nucleus (12th cranial nucleus), and the inferior olive, NK, SK, E, and K binding sites are expressed in high concentrations in the nucleus of the solitary tract. See Figure 1 for explanation. Scale bar, 1 mm.

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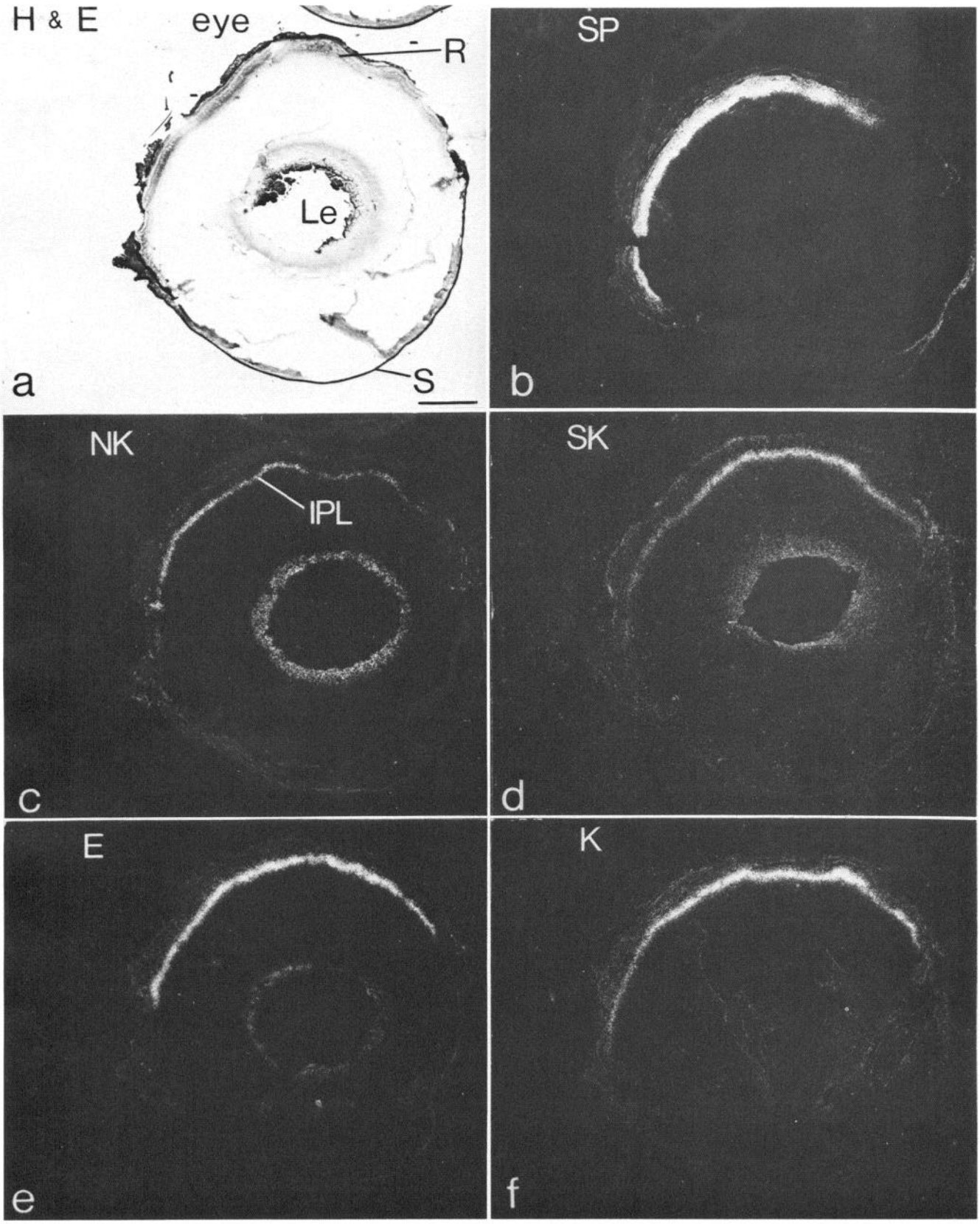
Figure 7. Autoradiographic localization of tachykinin receptor binding sites in coronal sections in rat eye. In Figures 7–10, *a* is a light-field photomicrograph of a rat peripheral tissue section that has been stained with hematoxylin and eosin (H&E) to aid in the orientation of the tissue



section, whereas *b–f* are dark-field autoradiograms of adjacent sections that demonstrate the distribution of binding sites for Bolton-Hunter labeled: *b*, substance P (SP); *c*, neuromedin K (NK); *d*, substance K (SK); *e*, eledoisin (E); and *f*, kassinin (K). In the photomicrographs *b–f*, the light areas correspond to high concentrations of binding sites. Unless otherwise noted, all white areas correspond to specific binding sites in that the binding sites were absent in adjacent sections where an excess (10^{-6} M) of the appropriate cold peptide was added to the incubation medium. Note that, whereas specific SP, NK, SK, E, and K binding sites are localized to the inner plexiform layer, the binding sites associated with the lens are not displaced in the presence of 10^{-6} M cold peptide and are therefore nonspecific binding sites. Scale bar, 1.0 mm.

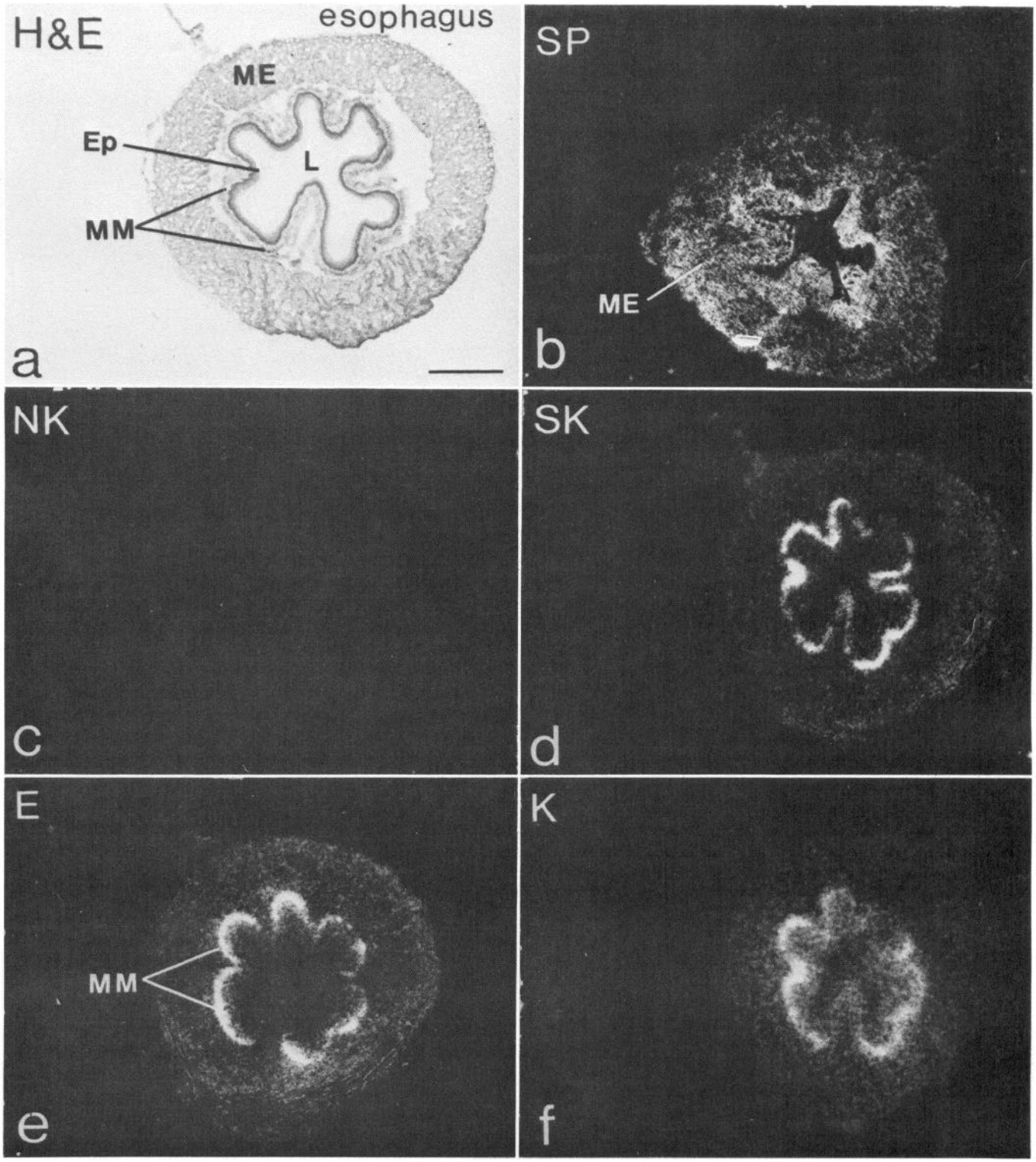


Figure 8. Autoradiographic localization of tachykinin receptor binding sites in a transverse section of the esophagus. Note that, whereas SP binding sites are expressed by the external muscle layer of the esophagus, SK, E, and K binding sites are expressed in moderate concentrations by the muscularis mucosae. Also note that no specific NK binding sites could be detected. Scale bar, 1.0 mm.

Discussion

Tachykinin receptor classification

Receptor classification for classical transmitters has been based largely on the use of specific high-affinity antagonists; but for the tachykinins, no such antagonists have yet been discovered or produced. Thus, tachykinin receptors have been classified in the past mainly on the use of agonists in pharmacological experiments (Lee et al., 1982, 1986); recently, radioligands have also been employed (Torrens et al., 1983, 1984, 1985; Viger et al., 1983; Beaujouan et al., 1984, 1986; Cascieri and Liang, 1984; Ninkovic et al., 1984; Cascieri et al., 1985; Maggio et al.,

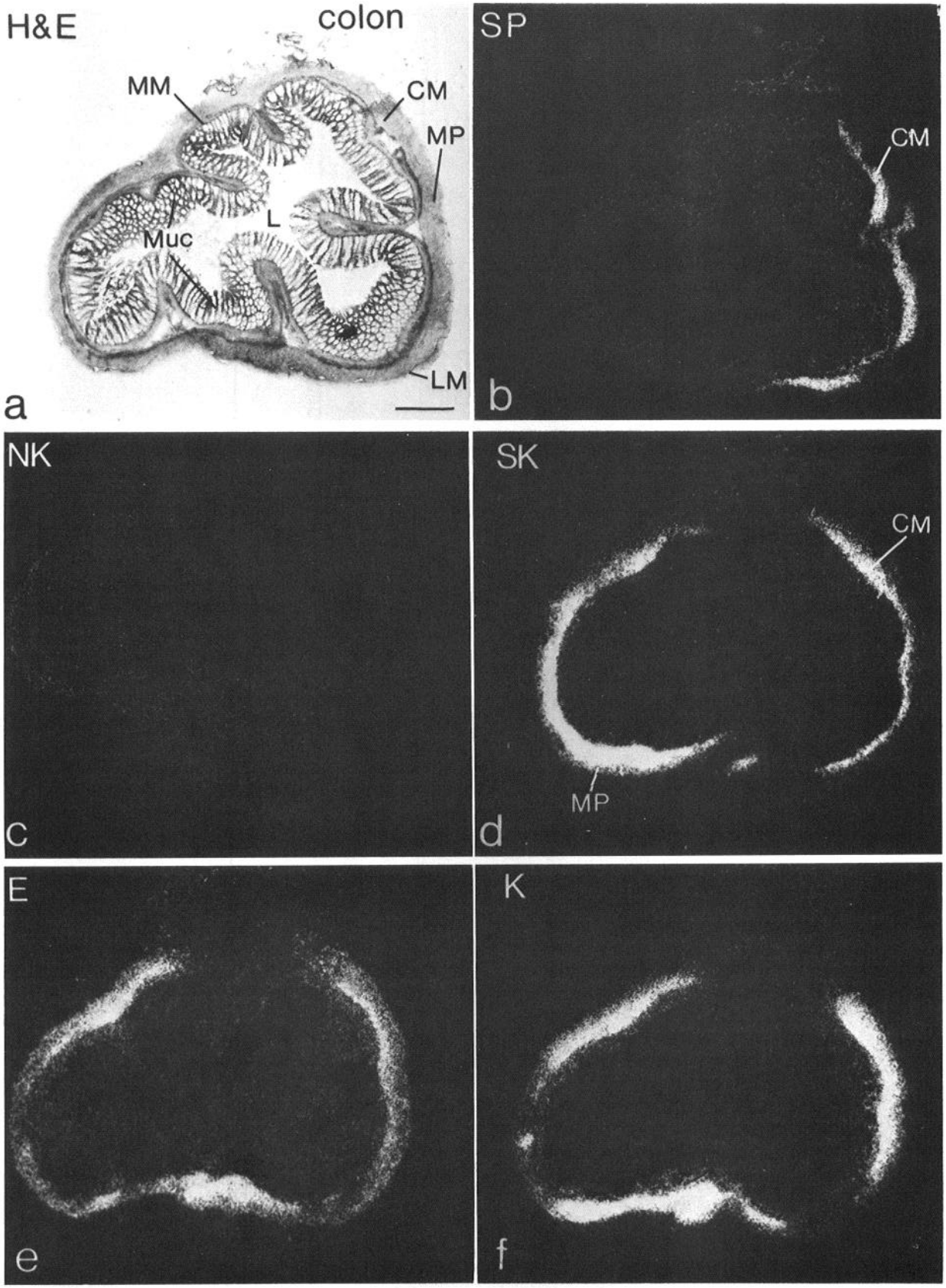


Figure 9. Autoradiographic localization of tachykinin receptor binding sites in transverse sections of the rat colon. Note that, while SP, SK, E, and K binding sites are expressed in moderate concentrations by the external circular muscle, no specific NK binding sites could be detected. Scale bar, 1.0 mm.

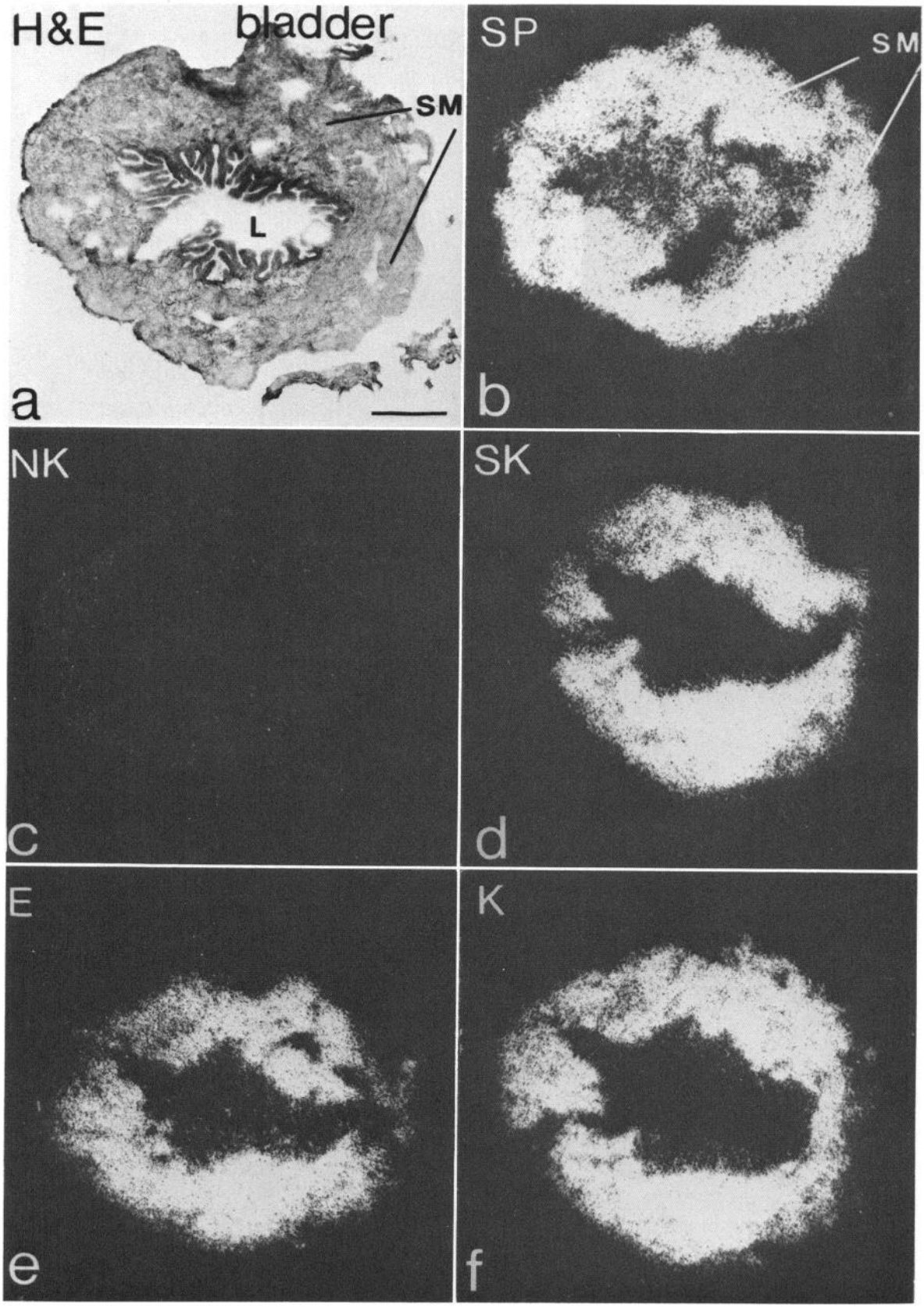


Figure 10. Autoradiographic localization of tachykinin receptor binding sites in transverse sections of the rat bladder. Note that, whereas SP, SK, E, and K binding sites are expressed by the external muscle, no NK binding sites could be detected. Scale bar, 1.0 mm.

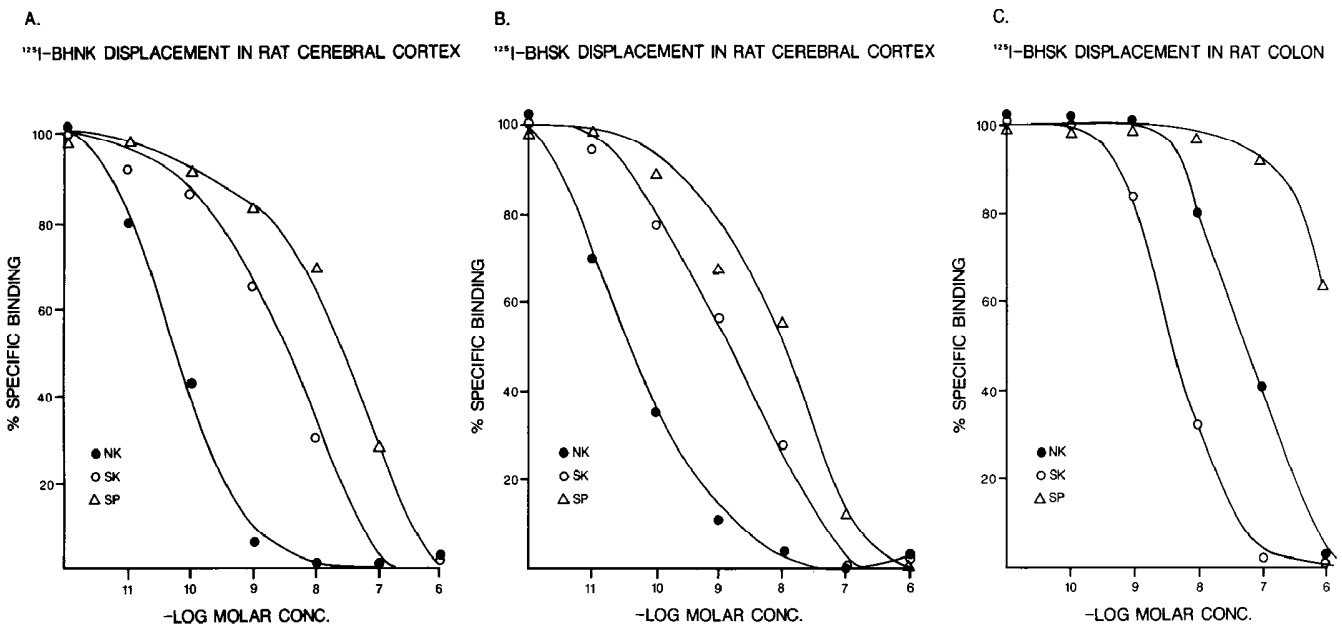


Figure 11. *A*, Inhibition curves for ^{125}I-BHNK binding to laminae 4 and 5 of the somatosensory area of the cerebral cortex. Slides were incubated with ^{125}I-BHNK with graduated concentrations of unlabeled NK, SK, or SP. The optical densities were determined for the high-density binding sites and plotted against the concentration of cold peptide present. *B*, Inhibition curves for ^{125}I-BHSK binding in laminae 4 and 5 of the somatosensory area of the cerebral cortex. *C*, Inhibition curves for ^{125}I-BHSK binding in the circular muscle of the rat colon. The data are from a representative experiment. Each point represents the mean of a triplicate determination where the SE is less than 15% of the mean.

1985; Bergstrom et al., 1987). Each method is based on assumptions that are difficult to verify experimentally (Maggio, 1988); as a result, tachykinin receptor nomenclature has been a matter of active discussion and dispute in recent years (Henry et al., 1987). Some of the nomenclature systems currently in use are given in Table 4; since different nomenclature systems are defined by different criteria in different systems, the equivalences implied in Table 4 are only approximate.

In the past, when SP was believed to be the only mammalian tachykinin, the most popular tachykinin receptor classification (Lee et al., 1982) divided mammalian peripheral tissues into 2 categories based on agonist potency. In one group of tissues, the tachykinins tested were roughly equipotent; in the other group of tissues, tachykinins bearing a branched aliphatic residue (Val, Ile) at position X in the defining sequence -Phe-X-Gly-Leu-Met-NH_2 were much more potent than the tachykinins with an aromatic (Phe, Tyr) residue at this position (Fig. 1). The tissues were said to contain SP receptors of the SP-P and SP-E types, respectively. While it is now clear that the mammalian tachykinin system of peptides and receptors is more complex than supposed at the time, the division of mammalian tachykinins and tachykinin receptors into 2 broad categories based on the nature (aliphatic or aromatic) of the amino acid at position X remains valid. The present study mapping the tachykinin binding sites of rat tissues using 14 tachykinin radioligands showing specific binding is consistent with these previous findings.

Most of the binding and autoradiographic studies on tachykinin receptors have employed peptides labeled with ^{125}I in the form of a Bolton-Hunter group, that is, a 3-[3-iodo-4-hydroxyphenyl]propionyl group on what was a free amino group in the native peptide. For a peptide the size of a tachykinin, that modification makes a considerable difference in the charge, size, and hydrophobicity, and the radioligand may not have the same receptor specificity as the native peptide. Indeed, it has been shown (Lee et al., 1986; Bergstrom et al., 1987) that addition of a Bolton-Hunter group can affect the receptor specificity of certain aliphatic tachykinins. Therefore, we have also employed radioligands other than the Bolton-Hunter conjugates in the present study. While NK is at present the leading candidate for physiological ligand at most aliphatic tachykinin binding sites in the CNS and SK the leading candidate at most aliphatic tachykinin binding sites in peripheral tissues, there remain many uncertainties. For example, both binding and pharmacological experiments have made it clear that tachykinin receptors are not fastidiously specific, and cross-talk between ligands and receptors in this neuropeptide family may occur *in vivo* as well as *in vitro*.

SP-preferring receptor

In the present report we have shown that there are at least 3 types of tachykinin binding sites in the rat. The first type of binding site, referred to as the SP site, is found in both the brain and periphery. Displacement experiments demonstrate that SP is approximately 1000 times as potent as either SK or NK at displacing BHSP in both the brain (rat cerebral cortex) and the periphery (canine chief cells, unpublished observations), and thus this binding site appears to be relatively specific for SP. These results are in general agreement with what is known about the distribution of SP. Authentic SP appears to be present in both the brain and peripheral tissues (Chang and Leeman, 1970; Nilsson et al., 1975; Franco et al., 1979; Maggio et al., 1983; Kanazawa et al., 1984; Maggio, 1985; Deacon et al., 1987) and should be available to occupy the SP binding site. The question of whether there are subtypes of SP receptors in the brain and periphery remains open. In the present experiments we have not addressed this question, for while possible evidence for the existence of subtypes of SP receptors has been presented, none of the techniques used to address the question are definitive since possible artifacts due to differences in degrading enzymes and receptor accessibility cannot, at present, be ruled out.

NK-preferring receptor

The second type of binding site which we have called the NK site is found only in the CNS. Displacement experiments (Fig. 11*A*) suggest that NK is approximately 10 times as potent as SK and 1000 times as potent as SP in displacing BHNK from binding sites in the brain (cerebral cortex), and NK is also more potent than either SK or SP in displacing BHSK (Fig. 11*B*) from its binding site. We could not detect this type of receptor in any peripheral tissue we examined; i.e., we could not find any specific binding sites for any NK ligand in any rat peripheral tissues we examined, or in any segment of the canine (Mantyh et al., 1988b) or human GI tract (Mantyh et al., 1988a). This NK binding site also appears to be labeled by kassinin and eledoisin radioligands since these amphibian (K) and molluscan (E) tachykinin analogs labeled a similar set of regions throughout the rat brain. While NK is somewhat more potent than SK in displacing either BHNK or BHSK, both NK and SK are present in the brain (Kangawa et al., 1983; Kimura et al., 1983; Maggio et al., 1983; Kanazawa et al., 1984; Minamino et al., 1984). Since we do not know the actual concentration of peptide at the receptor binding sites we feel it is premature to rule out SK as a possible endogenous ligand for this site.

SK-preferring receptor

The third type of receptor binding site is found only in peripheral tissues. We have referred to it as the SK binding site since SK appears to be the endogenous ligand and is the most potent tachykinin analog in displacing BHSK. Thus, in the colon SK is approximately 35 times as potent as NK and 1000 times as potent as SP in displacing BHSK from the circular muscle of the colon. One additional reason for referring to this receptor as SK is that it has been reported (Deacon et al., 1987) that immunoreactive NK is not detectable in guinea pig GI tissue. However, mRNA for NK has been detected in similar tissues in cow (Kotani et al., 1986). The explanation for this apparent paradox is unknown. However, in light of the displacement experiments, the presence of BHNK binding sites in brain but not in peripheral tissues, and the presence of detectable NK in the brain but not in peripheral tissues, we have tentatively named this peripheral tachykinin receptor SK. The specificity of this peripheral bovine SK receptor has recently been examined in a frog oocyte expression system (Masu et al., 1987) and the amino acid and nucleotide sequence of this receptor described (Harada et al., 1987). These data are in agreement with the present data in that the SK receptor is expressed in peripheral tissues and shows a preference for SK.

Physiological functions of tachykinin receptors

While it appears from the present result that there are at least 3 different types of tachykinin receptors in the rat, determining which physiological functions these receptor binding sites may mediate, especially in the brain, is less clear. The major reason for the difficulty in assigning any specific neuronal function is the complexity of the brain, but of considerable importance is the degree of resolution obtainable with the autoradiographic technique. For example, while spinal motor neurons are sufficiently large and well delineated that specific binding sites have been shown to be associated specifically with these neurons (Helke et al., 1984; Charlton and Helke, 1985) in other areas such as the striatum, where neurons, glia, and fibers of passage are intermixed, it is difficult to determine exactly which cell type expresses the binding site. This difficulty is underlined by the observation that glia have been shown to express high concentrations of SP binding sites both in culture (Torrens et al., 1986; Shults et al., 1987) and *in situ* after neuronal injury (Zimmerman et al., 1988). Thus, in most areas of the brain (i.e., cerebral cortex, amygdala, striatum) while one assumes that the tachykinin receptors are expressed predominately by neurons, it is possible that glia also express tachykinin receptors in these brain areas. In some areas of the brain stem and spinal cord, a defined role for SP and NK receptors is beginning to emerge. Whereas SP receptors are concentrated in both motor and sensory areas, the NK binding sites appear to be associated almost exclusively with the sensory system. Thus, SP binding sites are present over brain-stem motor nuclei 3, 7, 10, 12, and around ventral spinal motor neurons, preganglionic sympathetic neurons and Onuf's nucleus (Helke et al., 1984). No detectable concentration of NK binding sites is associated with these areas. In contrast, in sensory areas of brain stem and spinal cord (such as laminae 1 and 2 of the spinal trigeminal nucleus pars caudalis and laminae 1 and 2 of the spinal cord), both SP *and* NK receptors are present in high concentrations. Since incoming peptide containing C-fibers terminate heavily in these laminae, it is assumed that these binding sites are involved in processing incoming sensory information.

While it is difficult to define a function for tachykinin binding sites in the CNS, both the present study and previous pharmacological studies suggest that tachykinins play an important regulatory role in a wide variety of peripheral tissues (Pernow, 1983). One of the best-established functions for SP in the peripheral tissues is its potent *spasmogenic* effects on several peripheral smooth muscles. In the present study, we have demonstrated that SP and SK binding sites are present over smooth muscle in the esophagus, colon, and bladder. In the esophagus the smooth muscle of the muscularis mucosae expressed only SK binding sites and the muscularis externa SP binding sites, whereas in the colon and bladder both SP and SK binding sites are present over similar muscle layers. In the rat GI tract, SP and SK have repeatedly been shown to cause a potent contraction of the smooth muscle and are probably involved in gastric motility (Pernow, 1983). In the bladder, SP has been shown to be involved in the micturition response, which is apparently initiated by SP and SK released from visceral sensory nerves in which SP and SK are coexpressed (Maggio and Hunter, 1984) and presumably co-released. In addition, it has recently been demonstrated that binding sites for SP and SK can be differentially regulated since SP-preferring receptors, but not SK or NK, are ectopically expressed in high concentrations by arterioles, venules, and lymph nodules in human inflammatory disease affecting peripheral tissues (Mantyh et al., 1988a). These results support previous pharmacological experiments suggesting that tachykinins regulate a wide variety of peripheral actions, including gut motility and micturition, and there are at least 2 different tachykinin receptors in peripheral tissues whose expression appears to be independently regulated.

In summary, we have shown that there are at least 3 distinct tachykinin receptors detectable in rat brain and peripheral tissues. We have referred to these receptors as SP, NK, and SK according to the different ligands that preferentially bind to these binding sites, the inhibition constants displayed by the displacing peptides, and whether endogenous ligand is present to occupy the relevant binding site. Thus, SP receptors are found in both central and peripheral tissues, NK receptors in the CNS

only, and SK receptors in peripheral tissues. We feel that this is the lowest order of complexity for subdivision of tachykinin receptors and that further distinctions may be expected with the possible discovery of new mammalian tachykinins, the cloning of the tachykinin receptors, and the development of specific tachykinin analogs.

Appendix

Abbreviations used in the Figures

12	hypoglossal nucleus
aca	anterior commissure
ACg	anterior cingulate cortes
Arc	arcuate nucleus of the hypothalamus
AOP	anterior olfactory nucleus, posterior part
BH	Bolton-Hunter
BL	basolateral amygdaloid nucleus
BM	basomedial amygdaloid nucleus
CA	CA fields of Ammon's horn
Cb (10)	cerebellum, lobule 10
CG	central gray
Cl	claustrum
CM	external circular muscle
cp	choroid plexus
CPu	caudate-putamen (striatum)
ctx	cerebral cortex
DR	dorsal raphe
E	eledoisin
Ep	epithelium
FD	fascia dentata
fmi	forceps minor of the corpus callosum
FrPaM	frontal parietal cortex, motor area
FrPaSS	frontal parietal cortex, somatosensory area
IO	inferior olive
IPL	inner plexiform layer
K	kassinin
L	lumen
Le	lens
LM	external longitudinal muscle
Me	medial amygdaloid nucleus
ME	muscularis externa
MG	medial geniculate
MHb	habenula, medial part
MM	muscularis mucosa
MP	medial mammillary nucleus, posterior part
PHYS	physalaemin
R	retina
RF	rhinal fissure
S	sclera
SC	superior colliculus
SM	smooth muscle
SNC	substantia nigra, pars compacta
SNR	substantia nigra, pars reticulata
sol	solitary tract
SolM	nucleus of the solitary tract, medial part
Sp5I	nucleus of the spinal tract of 5, pars interpolaris
StrCtx	cerebral cortex, striate area
TK	tachykinin
VB	ventrobasal nucleus of the thalamus
VTA	ventral tegmental area

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