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Distribution of the neurotensin receptor NTS1 in the rat CNS studied using an amino-terminal directed antibody

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Abstract

The distribution of neurotensin receptor 1 immunoreactivity in the rat brain was studied using an antibody against the amino-terminal of the receptor expressed as a fusion protein with glutathione-S transferase. Affinity purified antibodies detected the fusion protein and the complete neurotensin receptor sequence expressed in Escherichia coli. The immunostaining was abolished by preabsorption with the amino-terminal fusion protein. Immunoreactive neurotensin receptor 1 immunoreactivity was detected on cell bodies and their processes in a number of CNS regions. In agreement with previous binding studies neurotensin receptor 1 immunoreactivity was particularly localised in cell bodies in the basal forebrain, nucleus basalis and substantia nigra. At the electron microscope level immunoreactivity was found both in axonal bouton and dendrites and spines in the basal forebrain indicating that neurotensin may act both pre- and post-synaptically. There were several regions such as the substantia gelatinosa, ventral caudate-putamen and the lateral reticular nucleus where the neurotensin receptor 1 positive cells had not previously been reported, indicating that distribution of this receptor is widespread.

Keywords: Neurotensin; Neurotensin receptor 1; Fusion protein; Dopamine

1. Introduction

The tridecapeptide neurotensin (NT) was originally isolated and sequenced from bovine hypothalamus (Carraway and Leeman, 1976; Leeman and Carraway, 1989). Subsequent studies using immunohistochemical and radio immunoassay techniques have shown that the peptide is widely distributed in both the central nervous system and the peripheral system (Carraway and Leeman, 1976; Kobayashi et al., 1977; Goedert et al., 1984a; Emson et al., 1985). In the central nervous system NT is known to influence dopaminergic transmission in the nigrostriatal and mesocorticolimbic pathways (Nemeroff, 1986; Kitabgi, 1989) and these NT–dopamine interactions implicate the neuropeptide in the pathophysiology of several CNS disorders including Parkinson's disease and schizophrenia (Garver et al., 1991; Rostene et al., 1992; Lambert et al., 1995). Several biochemical and behavioural properties of exogenously administered NT resemble those of classical antipsychotic drugs (Deutch and Zahm, 1992; Nemeroff, 1986; Kitabgi, 1989; Emson et al., 1985; Levant and Nemeroff, 1990). These led to the suggestion that NT may be an endogenous neuroleptic-like compound (Nemeroff 1980, 1986); however, the mechanism underlying the antipsychotic-like properties of NT remains unclear.

NT has also been shown to regulate acetylcholine release from rat cerebral cortex (Lapchak et al., 1990). Studies have also shown that NT can modulate glutamate release in the neostriatum (Ferraro et al., 1995) and that the peptide has an excitatory effect on the serotoninergic neurons of the dorsal raphe nucleus (Jolas and Aghajanian, 1996) and on the cholinergic neurons of the diagonal band of Broca and substantia innominata (Alonso et al., 1994). When injected into the CNS, NT has been reported to exert potent hypothermic and analgesic effects (Bissette et al., 1976; Clineschmidt et al., 1982).

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NT exerts its effects through two G-protein coupled receptors which may be linked to the inositol phosphate (Goedert et al., 1984a; Amar et al., 1986; Kanba and Richelson, 1987), cAMP (Bozou et al., 1986) or cGMP second messenger systems (Amar et al., 1987; Kanba and Richelson, 1987; Snider et al., 1986). A further novel type of neuropeptide receptor identical to gp95/sortilin, a 100-kDa protein with a single transmembrane domain, has recently been identified which may be particularly involved with the internalization of the peptide (see review by Vincent et al., 1999). The first neurotensin receptor (NTS1) was cloned from rat, (Tanaka et al., 1990) and subsequently from mouse and human cDNA (Vita, 1993; Watson, 1993), and corresponds to the high affinity NT binding sites demonstrated in the mammalian CNS. In addition to the high affinity neurotensin receptor (NTS1), which has been shown to be insensitive to the drug levocabastine (Tanaka et al., 1990), adult rat and mouse brain also contain a further lower affinity NT binding site which is sensitive to the antihistamine levocabastine and has a different regional distribution (Schotte and Laduron, 1987; Kitabgi et al., 1987). This evidence suggests that there are additional NT receptor subtypes in the CNS (Le et al., 1996) and a further distinct G-protein coupled neurotensin receptor (NTS2) was cloned from a rat hypothalamic cDNA library (Chalon et al., 1996) and from a rat and human brain cDNA library (Mazella et al., 1996). This receptor displays 64% homology at the nucleotide level and 43% amino acid identity to NTS1 (see recent overview by Vincent et al., 1999). This neurotensin receptor NTS2 was shown to correspond to the levocabastine sensitive low affinity receptor. NTS2 mRNA is found in neurones and glial in the rodent, particularly in the olfactory system, cerebral and cerebellar cortices and various hypothalamic nuclei (Walker et al., 1998). The distribution is distinct from that of NTS1 mRNA (Chalon et al., 1996; Vincent et al., 1999), which is exclusively neuronal, however, the two receptors (NTS1 and 2) overlap in various hypothalamic and septal nuclei. Most recently a further non G-protein coupled receptor (NTS3) has been reported; as noted earlier this NT binding receptor is identical to gp95/sortilin which has one transmembrane domain and a short cytoplasmic carboxy-terminal and may be involved in the uptake and degradation of NT (Petersen et al., 1997; Mazella et al., 1998; Nielsen et al., 1999). The CNS distribution of NTS3 has not yet been reported in detail but it is expressed in the forebrain of the mouse during development (Hermans-Borgmeyer et al., 1999).

In this study we report the distribution at the light and electron microscope level of the neurotensin receptor NTS1 immunoreactivity in the rat CNS using a polyclonal antibody recognising the amino-terminal of the NTS1 receptor. The use of the amino-terminal sequence allowed us to use a sequence which is not present in the related receptor (NTS2) and has no significant homology with sortilin (NTS3).

2. Materials and methods

2.1. Animals

Male Wistar rats from the Babraham colony with a body weight of 200–300 g were kept on a 12 h light/dark cycle with free access to food and water.

2.2. Chemicals

All chemicals were purchased from Sigma (Poole, UK), unless otherwise stated.

2.3. Expression of GST-Nter-NTR fusion protein

The DNA sequence corresponding to the *Bam* HI–*Apa* I fragment of the rat neurotensin receptor (NTS1) originally cloned into p-Malp-HMTX (pMalp-NTR-HMTX) was used to construct a fusion protein (Grisshammer et al., 1993; Tucker and Grisshammer, 1996). Fusion protein incorporating the entire predicted NH_2-terminus of the NTS1 receptor (amino acids 2–51) in conjunction with glutathione-S-transferase (GST) was expressed using the pGEX-system (Pharmacia, St Albans, UK). Expressed GST fusion protein was purified on a glutathione column (Pharmacia) and the purified protein was checked for its size and integrity by SDS-PAGE on a 10% gel and stained with Coomassie brilliant blue R. Protein concentrations were measured with a Bradford protein assay (Biorad, Hemel Hempstead, UK). GST-Nter-NTS1 fusion protein was subsequently used as the antigen for raising antibodies, for affinity purification of crude antisera and Western blotting.

2.4. Raising of polyclonal antibody against the NH_2 terminal neurotensin receptor NTS1

Polyclonal antisera were generated in rabbits by immunisation with the GST-Nter-NTS1 fusion protein. Fusion protein (100 μg) in Freund's complete adjuvant was injected intramuscularly followed by five booster injections of 100 μg in incomplete Freund's adjuvant at monthly intervals. A blood sample (20 ml) was taken after the third boost, clotted overnight at 4°C and the serum separated by centrifugation at 8000×*g*. Further blood samples were taken at monthly intervals and the serum separated and stored at −70°C for future use.

For the purification of the NH_2 terminal NTS1 anti-

bodies from whole rabbit serum, serum samples were diluted 1:100 in buffer B (100 mM Tris–HCl pH 8.0; 150 mM NaCl; 0.05% (v\v) Nonidet P-40) and subjected to micropurification as described by Harlow and Lane (1992). In brief, the diluted serum was incubated with 200 μg purified GST-Nter-NTS1 bound to a nitrocellulose filter (Sartorius, Göttingen, Germany) in buffer B with 0.5% w\v dried milk. Specific antibody bound to the GST-Nter-NTS1 was eluted from the nitrocellulose filters using 200 mM glycine, pH 2.5, and then neutralized with 2 M Tris pH 9.5. Affinity purified antibodies were stored at −20°C in 0.1 M PBS.

2.5. Western blotting

Samples containing 0.1 μg of the fusion protein or 5 μg of the bacterial pellet were separated by SDS-PAGE on a 10% gel. Gels were blotted onto 0.45 μm nitrocellulose filter (Sartorius, Göttingen, Germany) soaked in transfer buffer (50 mM Tris; 350 mM glycine: 20% v/v methanol; 0.038 w/v SDS) and blotted at 3 mA/cm^2 of gel for 1 h. The membrane was washed in 100 mM Tris–HCl pH 7.5; 150 mM NaCl; 0.1% v/v Tween-20 (TTBS buffer), blocked for 1.5 h at room temperature in TTBS supplemented with 3% BSA and incubated overnight at 4°C with primary antibody (1:500 dilution of the Nter-NTS1 antiserum).

The blot was washed three times in TTBS and incubated in a 1:2000 dilution of a peroxidase conjugated secondary antibody (anti-rabbit IgG, Sigma) for 1.5 h at room temperature. The blot was washed three times in TTBS and immunocomplexes were visualised using enhanced chemiluminescence detection (ECL, Amersham).

2.6. Immunohistochemistry

Rats (n=10) were deeply anaesthetised with Nembutal and perfused with 0.1 M PBS followed by 4% (w/v) neutral buffered paraformaldehyde in 0.1 M PBS in strict accordance with UK Home Office regulations and under provision of the appropriate animal licence. After perfusion, brain and spinal cord were dissected, postfixed for 2 h in 4% paraformaldehyde and transferred to 30% sucrose in 0.1 M PBS for 24 h at 4°C. Sledge microtome sections (30 μm) were cut and collected in 0.1 M PBS. Endogenous peroxidase was inactivated by incubating the sections for 10 min in 20% methanol, 1.5% H_20_2 in 0.1 M PBS and the sections were washed in 0.1 M PBS, 0.3% Triton X100 at room temperature. Sections were preincubated in 0.1 M PBS, 0.3% Triton, Bovine serum albumin 1 mg/l and normal goat serum 1.5% (buffer 1) for 30 min at room temperature and then incubated for 48 h at 4°C using the affinity-purified Nter-NTS1 antibody at a dilution of 1:10 in buffer 1. Sections were then washed in 0.1 M PBS; 0.3% Triton-X100 and incubated in secondary antibody (biotinylated anti-rabbit IgG; Vector Laboratories) 1:200 for 2 h at room temperature. After this, sections were washed again in 0.1 M PBS; 0.3% Triton-X100, incubated for 45 min in the ABC reagent (Vector Laboratories), then rinsed in 0.1 M PBS. The Nter-NTS1 signal was revealed using 0.5 mg/ml 3,3′ diaminobenzidine in a 0.001% H_2O_2 solution in PBS. Sections were mounted from distilled water onto 3-aminopropyltriethoxy-silane coated slides, and then dehydrated and treated with xylene before being coverslipped with DePeX medium (Gurr, BDH-Merck, Poole, UK). Absorption controls were carried out using 1 μM of the GST-Nter-NTS1 fusion protein overnight at 4°C to block the specific staining of the NTS1 antibody. Further controls included the omission of the primary antibody, and the use of pre-immune serum instead of primary antibody.

2.7. Electron microscopy

Female Wistar rats (200–250 g; Charles River, UK) were deeply anaesthetised with sodium pentobarbitone (Sagital, 60 mg/kg i.p.) and perfused transcardially with 50–100 ml of saline (0.9% NaCl) followed by 200 ml of the fixative (3% paraformaldehyde and 0.1–0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) with the aid of a peristaltic pump (flow rate of about 10 ml/min). The brain was quickly removed from the skull, sections (70 μm) were cut on a vibrating microtome and collected in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). In order to enhance the penetration of immunoreagents the sections were equilibrated in a cryoprotectant solution (PB, 0.05 M, pH 7.4, containing 25% sucrose and 10% glycerol), frozen in isopentane that was cooled in liquid nitrogen and then directly in liquid nitrogen. They were thawed in PBS. The immunocytochemistry was carried out essentially as described for light microscopy except that Triton X-100 was not included in the incubation media. On completion of the immunostaining the sections were post-fixed with osmium tetroxide solution (1% in 0.1 M PB at pH 7.4) for 20–30 min at room temperature. They were then washed in phosphate buffer, dehydrated in a series of increasing concentrations of ethanol and then treated with propylene oxide. After infiltration with resin (Durcupan ACM, Fluka) overnight, the sections were mounted on microscope slides, a coverslip applied and the resin cured at 60°C for 48 h. All sections were examined in the light microscope and the areas of interest were cut out from the microscope slide and glued to blank cylinders of resin for further sectioning. Ultrathin sections (silver/grey) were cut using an ultramicrotome (Reichert-Jung), collected on Pioloform-coated copper single slot grids,

stained with lead citrate and examined in an electron microscope (Philips CM10).

3. Results

3.1. Specificity of the antibodies

The affinity purified polyclonal antibody raised against the amino-terminal peptide of the neurotensin receptor NTS1 specifically detected a purified GST protein incorporating the amino terminus of the rat neurotensin receptor NTS1 on Western blots. No cross-reactivity was observed with the GST-C-terminal fusion protein, which incorporated the carboxyterminus of the rat neurotensin receptor NTS1 or to the GST-somatostatin receptor sst_1 and sst_{2a} fusion proteins, to a GST-μ opioid receptor fusion, to GST itself or to bovine serum albumin (Fig. 1a). The staining of the specific band for the GST-Nter-NTS1 protein at the expected size of 37 kDa was blocked by preabsorbing the antibody using 0.1 μM of the purified Nter-NTS1 fusion protein used for immunisation (data not shown).

Western blot analysis using *E. coli* cells expressing the full length NTS1 cloned into the pMalp-2 expression vector (Mazella et al., 1996) revealed an immunoreactive protein with a molecular mass of 95 kDa, corresponding to the expected size of the intact MalE-NTR1 fusion protein. In addition, lower molecular weight immunoreactive bands between 65 and 49 kDa were observed in the *E. coli* extracts, which are likely to reflect degradation of the full length receptor. No specific Nter-NTS1 positive bands were detected in *E. coli* cells expressing just the maltose binding protein and its tagged products derived from the unmodified vector (pMalp-2) (Fig. 1b).

3.2. Nter-NTS1 immunoreactivity in the brain

Rat brain sections immunostained with the affinity purified Nter-NTS1 antibody exhibited a selective pattern of staining. This staining was absent in sections incubated in the absence of the primary antibody, with preimmune serum (data not shown) or with purified antibody preabsorbed with 10 μM Nter-NTS1-GST fusion protein (Figs. 2b and 5b).

The basal forebrain showed a consistent pattern of NTS1-like immunoreactivity (Fig. 2a). Both neuronal perikarya and dendrites of a large group of neurones running from the diagonal band of Broca and the medial septum through the ventral pallidum were strongly stained (Fig. 2c, d). At higher magnification it was possible to visualise processes of stained neurones within these regions (Fig. 2f, g). Scattered nerve terminals were seen in the lateral septum. The bed nucleus of the stria terminalis was immunopositive, neurones were observed

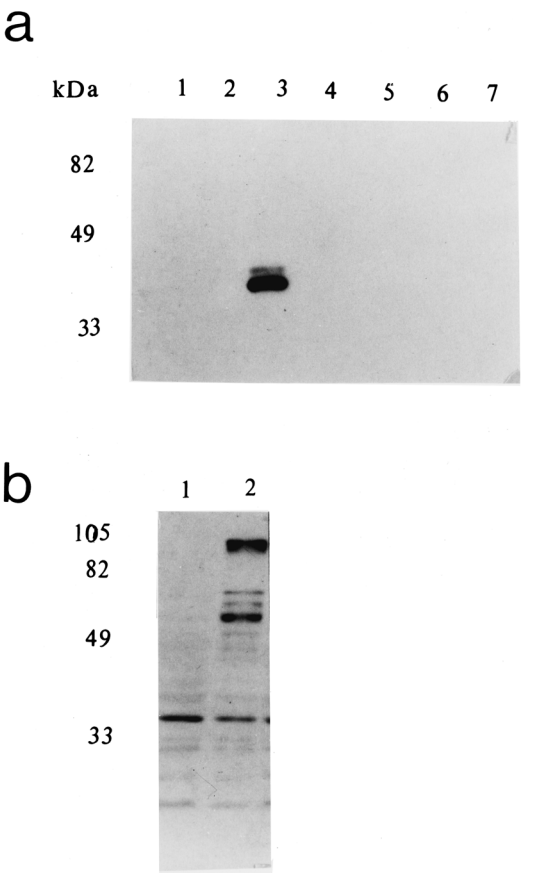


Fig. 1. Specificity of the neurotensin receptor antibody as revealed by Western blotting. (a) Western blot analysis showing the specificity of the affinity purified NTR receptor antibody (used at 1:500 dilution); 500 ng of bovine serum albumin (lane 1) or GST-fusion proteins incorporating the C-terminal fragment of the μ-opioid receptor (lane 2), the amino-terminal of the neurotensin receptor (lane 3), glutathione-S-transferase protein (lane 4), GST fusion proteins incorporating the carboxy-terminus fragment of the neurotensin receptor (lane 5), the somatostatin 2_a receptor (lane 6) and the somatostatin 1 receptor (lane 7) were separated by SDS-PAGE, blotted onto nitrocellulose membrane and immunodetection was performed as described. The NTR receptor antibody detected the predicted 37 kDa GST-Nter-NTR fusion protein, while no cross reactivity was observed with other GST fusion proteins or carrier protein. A Biorad prestained molecular-mass marker was used. (b) Western blot analysis showing the specificity of the amino-terminal neurotensin receptor antibody (used in 1:500 dilution) towards the whole neurotensin receptor. *E. coli* DH5α cells containing the plasmid pMalp-2 and the plasmid pMalp-NTR-HMTX were grown at 37°C and induced for 2 h with 2 mM IPTG and 2 mM NaN_3. Five micrograms of proteins of the bacterial pellet from the DH5α pMalp2 (lane 1) and from the DH5α pMalp-NTR-HMTX (lane 2) were separated on a SDS-PAGE, blotted on a nitrocellulose filter and immunodetection was performed as described. Note the major neurotensin receptor fusion protein specific band around 95 kDa. The lower molecular bands between 65 and 49 kDa represent degradation fragments of the neurotensin receptor fusion protein; lower molecular weight bands below 49 kDa are non-specific.

to extend fine processes in this region (Fig. 3a). Numerous strongly immunoreactive cell bodies and dendrites were observed in the substantia innominata and in the horizontal limb of the diagonal band of Broca. The

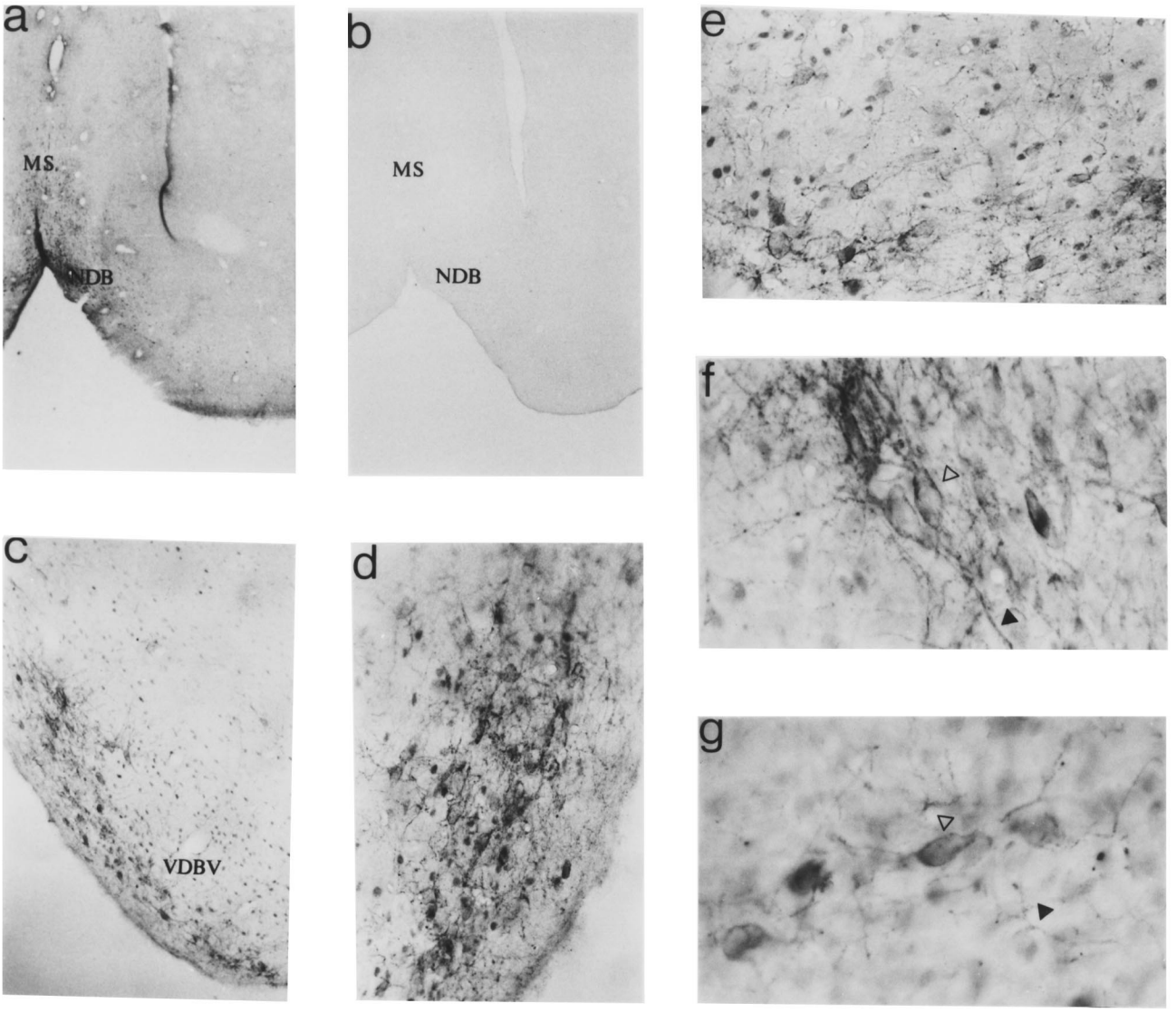


Fig. 2. Distribution of NTR1 immunoreactivity in the rat basal forebrain. (a) The medial septum and the nuclei of the diagonal band are strongly stained (magnification ×7) and (b) by comparison the preabsorbed control is devoid of immunoreactivity (magnification ×7). (c–f) In the ventral limb of the diagonal band, cell bodies (white arrows) and processes (black arrows) are positively stained (magnification ×100, ×250 and ×400, respectively). (g) Labelled cells and processes were also visualized in the medial septum (magnification ×400). Abbreviations: MS (medial septum), NDB (nucleus of the diagonal band), VDBV (nucleus of the vertical limb of the diagonal band, dorsal part).

Islands of Cajella were also stained. More sparse and less intensely reactive neuronal cells were also visible in the globus pallidus and in the lateral hypothalamic area. Immunopositive terminals were found in the caudate putamen, with few interneurones positively stained and in the ventral pallidum where perikarya and dendrites were strongly stained (Fig. 3c, d).

In the hypothalamus the supraoptic nucleus was strongly stained. Cell bodies and especially fibres were visualised, immunopositive fibres were also observed in the arcuate nucleus and in the suprachiasmatic nucleus and in the median preoptic nucleus. The amygdala contained weak labelled NTS1-like immunopositive neurones with few stained cell bodies and fine terminals, particularly located in the medial and central nuclei. Few regions of the thalamus demonstrated Nter-NTS1 immunoreactivity. Weak labelling was observed in the medial habenula, in the reticulus nucleus thalamus and a much stronger signal in the zona incerta where some cell bodies and dendrites were stained (Fig. 3b). The dorsal part of the endopiriform nucleus was positively stained. A weak and diffuse signal mainly associated with cell bodies was observed at the level of the entorhinal and piriform cortex (Fig. 3g, h) and in layer II of the olfactory tubercule. In other cortical fields a faint immunoreactivity was observed in layers II, III and V.

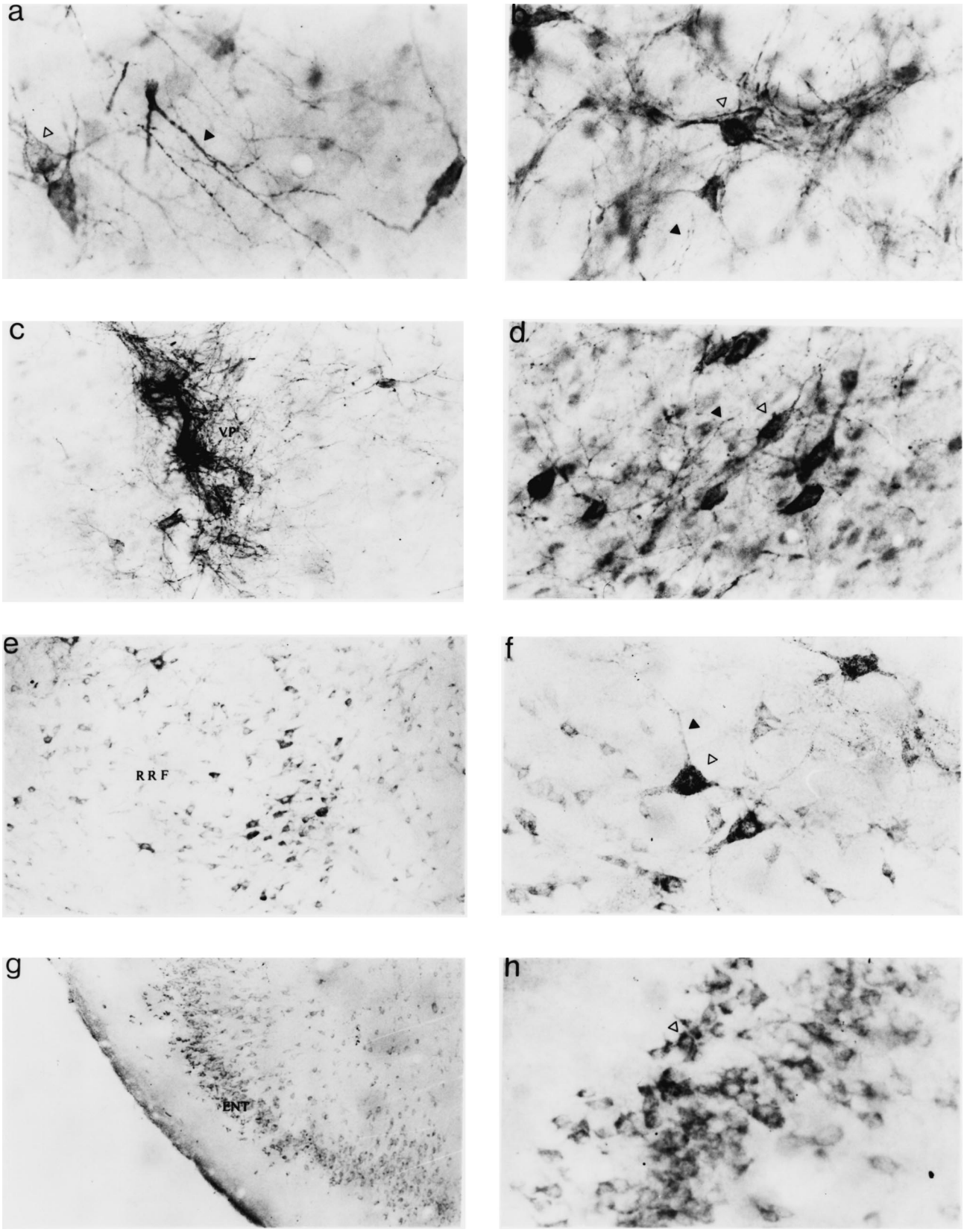


Fig. 3. High power magnification of NTR1 immunoreactive cells in different rat brain regions. (a) Bed nucleus of the stria terminalis (×400); (b) zona incerta (×400); (c, d) ventral pallidum (×250 and ×400); (e, f) retrorubral field (×250 and ×400); (g, h) entorhinal cortex (×250 and ×400). White arrows indicate cell bodies and black arrows indicate processes. Abbreviations: VP (ventral pallidum), RRF (retrorubral field), ENT (entorhinal cortex).

The forebrain sections that were incubated for electron microscopy were more weakly stained than those prepared for light microscopy as Triton X-100 was not included in the incubation media. Immunoreactive structures, identified by the brown amorphous diamnobenzidine (DAB) reaction product, in the ventral parts of the neostriatum and the pallidal complex were selected for electron microscopic analysis. In the electron microscope the DAB reaction product was identified as an amorphous electron dense material that adhered to the external membranes of organelles including mitochondria and microtubules, and the internal plasma membrane (Fig. 4a–e). Most of the immunoreactive structures in the neostriatum were identified as dendrites (b) or spines (a) and only rarely axonal boutons (c). In the globus pallidus and the ventral pallidum, immunoreactive structures were identified as dendrites that were typically postsynaptic to many terminals forming symmetrical synapse (Fig. 4d, e).

In the midbrain, labelling was found in the substantia nigra and in the ventral tegmental area (VTA) (Fig. 5a, c). In the substantia nigra pars compacta a very strong signal was associated with the perikarya and with their processes (Fig. 5e–g). Labelled cells were also observed in the pars reticulata and to a lesser extent in the pars lateralis. The VTA was strongly immunoreactive, with cells and their processes labelled (Fig. 5h). In the retrorubral field, cell bodies with primary dendritic branches were positively stained (Fig. 3e, f). In the superior colliculus a faint fibre signal associated with the zonal layer was observed. A few cell bodies and fibres were positively stained in the periaqueductal gray. Highly arborized, densely immunoreactive cell bodies and dendrites were observed in the brainstem within the lateral reticular nucleus (Fig. 6c, d). A few cell bodies were also observed outside the lateral reticular nucleus in the vestibular nuclei. In the spinal cord NTS1-like immunoreactivity was found exclusively in the dorsal horn (Fig. 6a). The lamina II (substantia gelatinosa) contained a very dense plexus of immunoreactive fibres (Fig. 6b) with cell bodies in the deep part of layer II.

4. Discussion

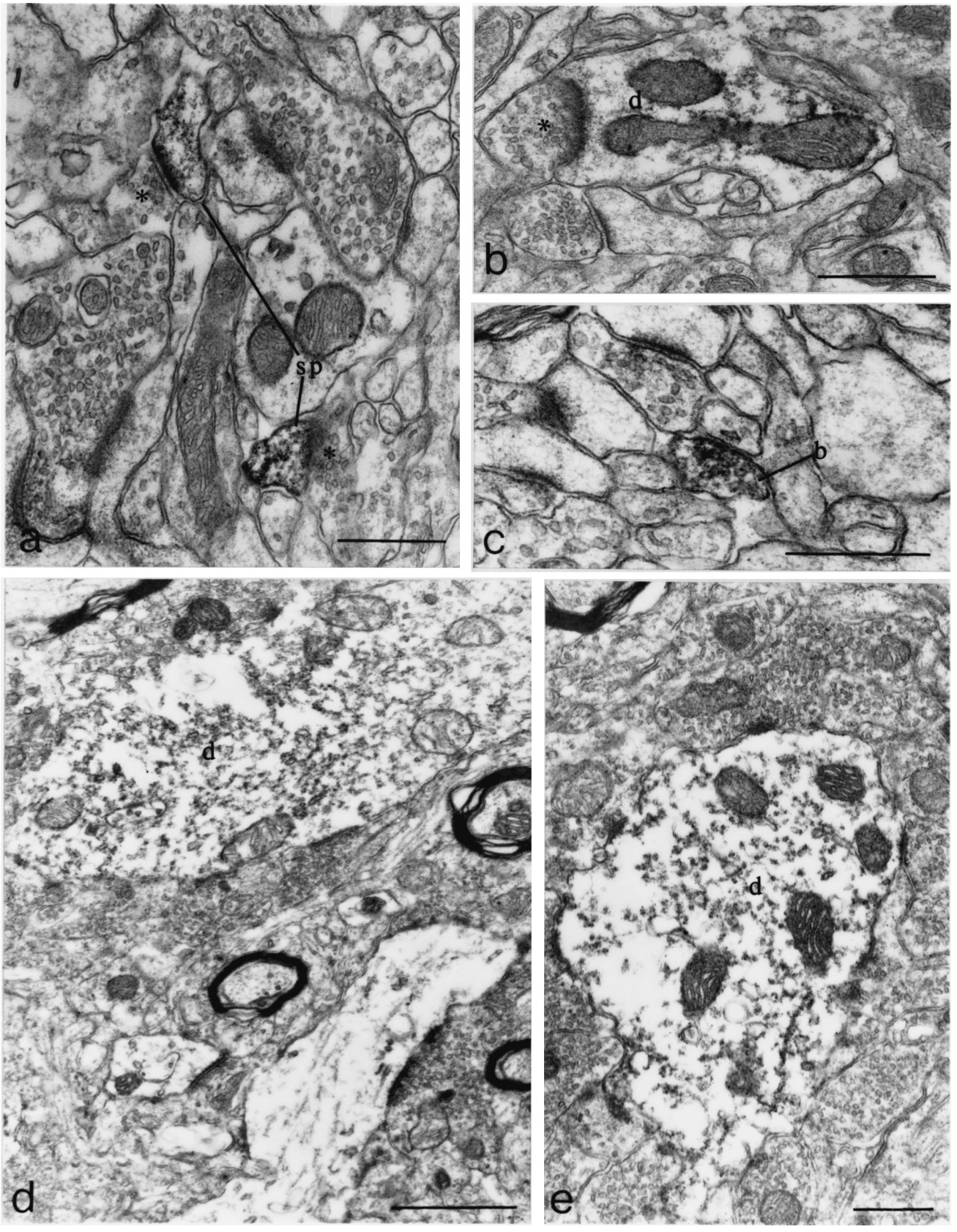
The present study provides a detailed description of the regional, cellular and sub-cellular distribution of immunoreactivity for the neurotensin receptor NTS1 in the mammalian CNS using a novel antiserum against the amino-terminus of the levocabastine-insensitive rat neurotensin receptor NTS1 (Tanaka et al., 1990; Vita, 1993). This NTS1 sequence has only minimal similarity to that of the amino-terminal of the levocabastine-sensitive neurotensin receptor site (NTS2) with only four out of 50 amino acids in common. (Chalon et al., 1996; Mazella et al., 1996) and no significant homology with NTS3/sortilin (Vincent et al., 1999).

The specificity of the polyclonal NTS1 antibody used here was established by Western blotting. The antibody specifically recognised the GST-fusion protein incorporating the amino-terminus of the NTS1, whilst the carboxy-terminus neurotensin receptor NTS1-GST fusion protein and several other heterologous proteins, including BSA and sst receptors were not recognised. In addition, the antibody specifically recognised the whole rat NTS1 receptor protein expressed in pMalp-2. The antibody detected a protein band at 95 kDa, in keeping with the molecular weight reported for the tagged MalE-NTS1 fusion protein detected using the monoclonal antibody 9E10, which recognises the tag or the polyclonal anti-maltose binding protein antibodies (Grisshammer et al., 1993; Tucker and Grisshammer, 1996) that are specific for the original unmodified expression vector.

The regional distribution of the amino-terminal NTS1 immunoreactivity reported here for the rat brain was in good agreement with that of the NT binding sites previously documented by receptor autoradiography (Quiron et al., 1982; Uhl and Snyder, 1977; Young and Kuhar 1979, 1981; Goedert et al., 1984b). Good agreement was also found with the distribution of NTS1 mRNA as studied by in situ hybridisation techniques (Sato et al., 1992; Elde et al., 1990; Nicot et al., 1994), the polymerase chain reaction (RT-PCR) (Mendez et al., 1997), and the distribution of NTS1 immunoreactivity (reported by Boudin et al. 1996, 1998).

A high percentage of both NTR1 mRNA expression and of NT binding sites was found in the mesenchephalic region of the substantia nigra and VTA, and binding studies coupled to immunohistochemical and lesion studies have shown that NT receptors are associated with dopaminergic neurones (Swanson, 1982; Szigethy and Beaudet, 1989; Mauo et al., 1990; Palacios and Kuhar, 1981). Similarly in our results these regions showed a strong NTS1 immunoreactivity associated with

Fig. 4. Electron micrographs of neurotensin-immunoreactive structures in the ventral part of the neostriatum (a–c), the globus pallidus (d) and the ventral pallidum (e). (a) Two spines (sp) contain the electron dense DAB reaction identifying them as immunoreactive for the neurotensin receptor. The spines are postsynaptic to non-reactive boutons (asterisks) forming asymmetric synapses. Other spines that do not display immunoreactivity are present in the field. (b) A neurotensin receptor-immunoreactive dendrite (d) containing the DAB reaction product that has adhered to the outer membrane of mitochondria. An immunonegative bouton (asterisk) forms asymmetrical synaptic contact with the dendrite. (c) A vesicle-containing bouton (b) that displays immunoreactivity for the neurotensin receptor. Note the immunonegative synaptic bouton directly above the labelled bouton. (d, e) Immunoreactive dendrites (d) in the globus pallidus (d) and the ventral pallidum (e). The dendrites show typical features of pallidal dendrites and are ensheathed by many axonal boutons. Note the immunonegative dendrites in the lower half of micrograph d. Scales: (a, b), same magnification bar=0.5 μM; (c), 0.5 μM; (d), 1 μM; (e), 0.5 μM.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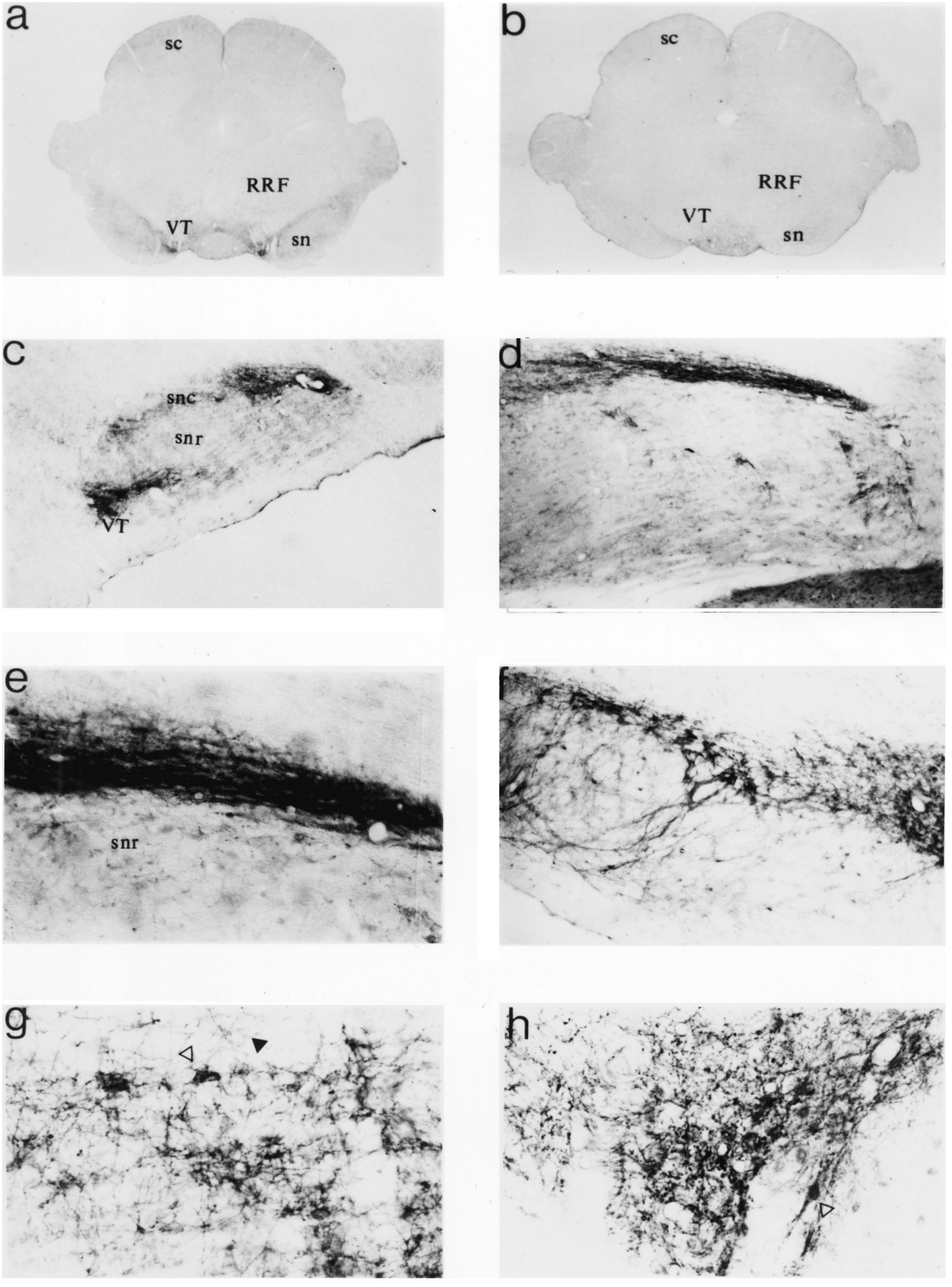
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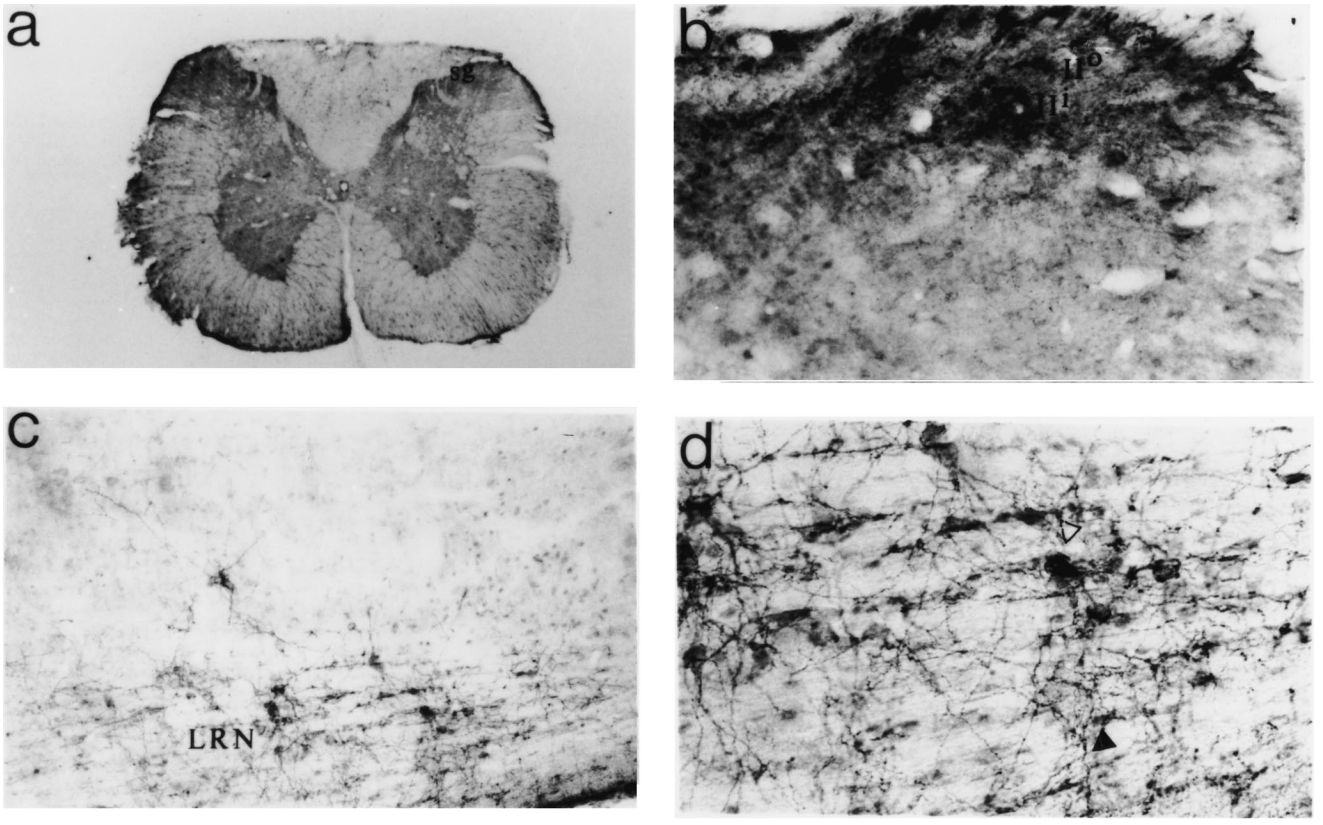

Fig. 6. NTS1 in the spinal cord and brain stem. NTS1 immunoreactivity is localised in the substantia gelatinosa layers II^o and II^i in the spinal cord (a, b). Note the strongly labelled neurones in the lateral reticular nucleus (c, d).

both cell bodies and processes, leading us to conclude that the dopaminergic neurones of the ventral mesencephalon express the NTS1 receptor and that this NT receptor subtype is the major NTR implicated in the reported NT dopamine interaction in these regions. Several projection areas from dopaminergic mesencephalic neurones were also immunopositive for the NTS1. These projection areas include caudate-putamen, the bed nucleus of the stria teminalis, the olfactory tubercule, for the mesostriatal dopamine system, as well as the lateral septum, the amygdala, the lateral habenula, the piriform, enthorinal and cingulate cortices for the mesolimbic and the mesocortical dopamine pathways. These projection areas have been found to contain NT binding sites but lack NTS1 mRNA (Elde et al., 1990; Sato et al., 1992; Nicot et al., 1994; Alexander and Leeman, 1998). In some of these areas, such as the bed nucleus of the stria terminalis, the amygdala and the ventral striatum, we visualised not only nerve terminals but also cell bodies suggesting that the absence of a NTS1 mRNA signal in these areas may be due to the limitations of the sensitivity of the in situ procedure used. Consistent with this suggestion, electron microscopy localized the NTS1 immunoreactive to dendrites and spines of intrinsic neurones. In the pallidum, NTS1 immunoreactivity was found in dendrites postsynaptic to symmetrical, presumptively inhibitory terminals.

Many NTS1 mRNA containing cells associated with NT binding sites have been observed in the rat forebrain (Elde et al., 1990; Sato et al., 1992; Nicot et al., 1994; Alexander and Leeman, 1998) and in accordance with these results the Nter-NTS1 antibody used here recognised a large group of cell bodies and processes in the basal nucleus, substantia innominata and the medial septum diagonal band complex. Previous studies indicate that a large population of neurones showing NT binding sites are acetylcholinesterase positive in the rat forebrain and by implication these are likely to be NTR1 positive

Fig. 5. Distribution of NTS1 immunoreactivity in the rat midbrain. (a) Note the staining of the substantia nigra, ventral tegmental area and retrorubral field and the weak staining in the superior colliculus (×7). (b) All specific staining was displaced when the antibody was preadsorbed with the Nter-NTR fusion protein leaving only a non-specific staining in the interpeduncular nucleus (×7). (c–e) Within the substantia nigra the pars compacta was particularly stained (×25, ×100 and ×250, respectively), (f, g) where both cell bodies and processes were immunopositive (×250 and ×400, respectively). (h) Strong staining was also observed in the ventral tegmental area where perikarya and fibres were immunopositive (×400). White arrows indicate cell bodies and black arrows neuronal processes. Abbreviations: RRF (retrorubral field), sc (superior colliculus), sn (substantia nigra), snc (substantia nigra pars compacta), snr (substantia nigra pars reticulata), VT (ventral tegmental area.

cholinergic cells (Alonso et al., 1994; Boudin et al., 1996). NT has been shown to modulate ACh release from the rat cerebral cortex and this effect was abolished by quinolinic acid lesions of the basal forebrain neurones (Lapchak et al., 1990), which removed the ascending cholinergic projections.

The hypothalamus has been shown to contain a high density of NT binding sites, whereas a weak NTS1 mRNA was observed associated with the suprachiasmatic nucleus and the dorsomedial hypothalamic area (Nicot et al., 1994). The NTS1 immunoreactivity in the hypothalamic area observed with antibody was distributed in a number of these nuclei including the arcuate nucleus, the supraoptic nucleus and the suprachiasmatic nucleus, moreover, a faint and diffuse immunosignal was observed in the lateral hypothalamic area. The median eminence, a hypothalamic area that is enriched in NT binding sites, has been observed to contain immunopositive NTS1 neurones. The relative lack of NTS2 mRNA in the hypothalamus (Mazella et al., 1996; Sarret et al., 1998) suggests that the various neuroendocrine effects of NT are likely to be mediated by the NTS1 receptor on hypothalamic cells including the tuberoinfundibular dopamine cells.

In the spinal cord NT binding studies have shown a high concentration of specific NT binding sites in the substantia gelatinosa of the spinal cord. These binding sites seem likely to include receptors intrinsic to the substantia gelatinosa as well as others derived from dorsal root ganglia which express NTS1 mRNA. Our finding of NTS1 immunopositive neurones and in immunoreactivity in the substantia gelatinosa suggest that at the spinal level the NT antinociceptive action may be mediated by the NTS1 receptor. The NTS1 immunoreactivity on cells in the brain stem may also reflect the involvement of NT in sensory processing at this level.

In summary we provide a detailed study of the distribution of the neurotensin NTS1 receptor protein in the rat CNS using a novel antiserum which is specific for the amino-terminal of this receptor type. The staining pattern was in good agreement with major sites of radiolabelled NT binding suggesting that this receptor is the isoform mediating the effects of NT on the dopamine and cholinergic systems and some of the antinociceptive effects of NT.

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