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Neurotensin and receptors

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Keywords

degradation

dopamine systems

eating behaviour

metallo-endopeptidases

neuromedin N

neurotensin

neurotensin receptors

pain

pro-hormone convertases

pro-neurotensin/neuromedin N

Synopsis

Neurotensin (NT) is a neuropeptide predominantly expressed in the brain and the gut. The peptide exerts its effects through two G-protein-coupled receptors, designed as NTS1 and NTS2, and through a single transmembrane domain protein, called NTS3 or sortilin, that belongs to a small family of sorting receptors. In the brain, NT and its receptors are distributed in a regionalized fashion, and the peptide modulates the activity of neuronal systems involved in behavioral responses such as locomotor activity, rewarding, drug seeking, eating, and pain sensation.

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Neurotensin and Receptors

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Neurotensin receptors

Pain

Prohormone convertases

Proneurotensin/neuromedin N

Introduction

Neurotensin (NT) is a tridecapeptide ([Figure 1](#)) that was isolated in 1973 from bovine hypothalamus and shortly after from bovine small intestine. In the brain, NT is predominantly expressed in neurons within discrete brain areas and nuclei, where it acts as a neuromodulator. The effects of centrally administered NT include the well-documented interaction of the peptide with dopaminergic (DA) systems, the ability to

induce opioid-independent analgesia, inhibition of food intake, and modulation of pituitary hormone release. In the periphery, NT is primarily produced in endocrine N cells scattered throughout the jejuno-ileal mucosa. The peptide is released on food ingestion and regulates a number of digestive processes, including gastrointestinal motility and pancreatic and biliary secretion. In addition, NT exerts growth-promoting effects on normal gastrointestinal tissues and on cancer cells derived from a variety of peripheral organs. Other organs that produce NT include the heart and adrenals. Like all neuropeptides, NT is synthesized as part of a larger precursor that also contains neuromedin N (NN), a NT-like hexapeptide. Pro-NT/NN is differentially processed in tissues, where it is expressed to give rise to different combinations of active peptides. NT and NN are released from neuroendocrine cells in a Ca^{2+} -dependent manner and exert their effect by interacting with NT receptors located on target cells, of which three have been cloned to date. Two, the NTS1 and NTS2, belong to the family of G-protein-coupled receptors with seven transmembrane domains. The third one, NTS3, is identical to sortilin, a protein that belongs to a family of receptors with one transmembrane domain and is involved in the sorting of proteins to various cell compartments. NT and NN actions are terminated by desensitization of the receptors and by degradation mechanisms that take place in the extracellular space. This article will review data pertaining mostly to the brain functions of NT.

Biosynthesis, Processing, and Degradation of NT

Cloning of canine and bovine pro-NT/NN complementary DNA revealed that NT and NN are part of the same precursor protein. Subsequently, cloning of the rat precursor gene showed it to comprise four exons and three introns, with NT and NN both being encoded in the fourth exon. Studies of pro-NT/NN gene induction in PC12 cells, a neuroendocrine cell line that synthesizes high amounts of pro-NT/NN in response to hormonal stimuli, showed that induction was under the coordinated control of several regulatory sequences, including activating protein-1, cyclic adenosine monophosphate (cAMP), and glucocorticoid response elements.

Rat pre-pro-NT/NN is a protein of 169-170 amino acids that starts with a signal peptide that is quickly removed on translocation into the endoplasmic reticulum to yield pro-NT/NN, whose organization is depicted in [Figure 1](#). The precursor then travels through the regulated secretory pathway, where it undergoes several post-translational transformations that end with the storage of the final maturation products in secretory vesicles of neuroendocrine cells. Rat pro-NT/NN contains four Lys-Arg dibasic sites (numbered 1 to 4 from N- to C-terminus in [Figure 1](#)). Site 1 is located in the middle of the precursor whereas sites 2, 3, and 4 flank and separate NT and NN in the C-terminal portion of the precursor. In tissues that express pro-NT/NN, site 1 is generally unprocessed whereas sites 2, 3, and 4 are differentially cleaved, leading to the

production of different combinations of bioactive products represented in [Figure 1](#). Thus, in the brain, pro-NT/NN gives rise to NT and NN. In the gut, precursor cleavage leads mainly to the formation of NT and a large peptide ending with the NN sequence at its C-terminus (large NN). In the adrenals, NT, large NN, and a large peptide ending with the NT sequence (large NT) are the major products.

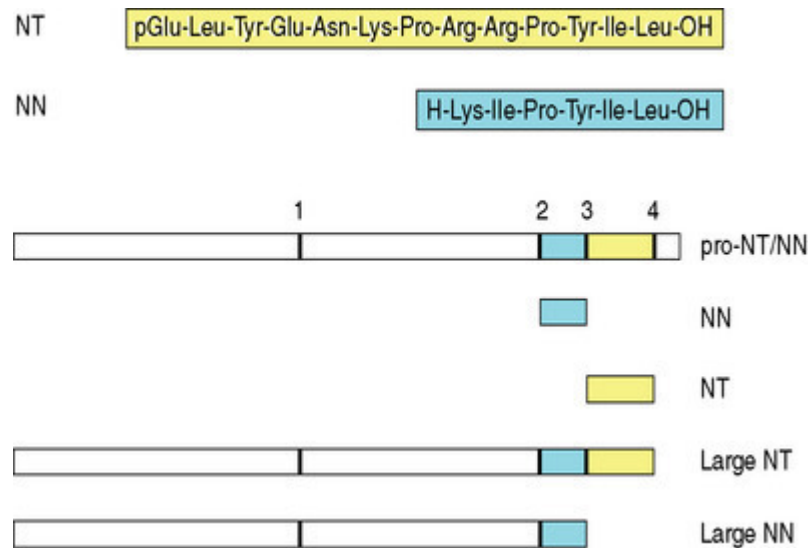


Figure 1. Amino acid sequence of NT and NN, organization of pro-NT/NN, and major bioactive products generated in brain and peripheral tissues by pro-NT/NN processing. The dibasic sites are represented by thick vertical bars and numbered from 1 to 4 from N- to C-terminal.

Processing

Dibasics are the consensus sites recognized and cleaved by processing endoproteases that belong to the identified family of proprotein convertases (PCs). PC1 and PC2 are exclusively expressed in the regulated secretory pathway of neurons and endocrine cells whereas PC5-A is expressed in both endocrine and non-endocrine cells. The cleavage specificity of the three PCs toward pro-NT/NN was studied in the neuroendocrine PC12 cell line that lacks PC1 and PC2 expression and presents very little PC5-A. As expected, wild-type PC12 cells poorly processed pro-NT/NN. In contrast, when stably transfected with either of the three PCs, each resulting transfectant readily processed the precursor, albeit with different patterns. Thus, in PC1-PC12 cells, cleavages at sites 3 and 4 proceeded almost to the same extent and were more efficient than cleavage at site 2. This predominantly generated NT and large NN and produced only small amounts of NN ([Figure 2](#)). In PC2-PC12 cells, pro-NT/NN was cleaved equally at sites 2, 3, and 4, thereby producing equivalent amounts

of NT and NN ([Figure 2](#)). In PC5-A-PC12 cells, site 4 was cleaved to a greater extent than site 3 whereas site 2 was very poorly processed, thus resulting in the production of NT, large NT and large NN in comparable amounts and of virtually no NN ([Figure 2](#)). It thus appears that PC1, PC2, and PC5-A process pro-NT/NN with patterns that are similar to those observed in the gut, brain, and adrenals, respectively.

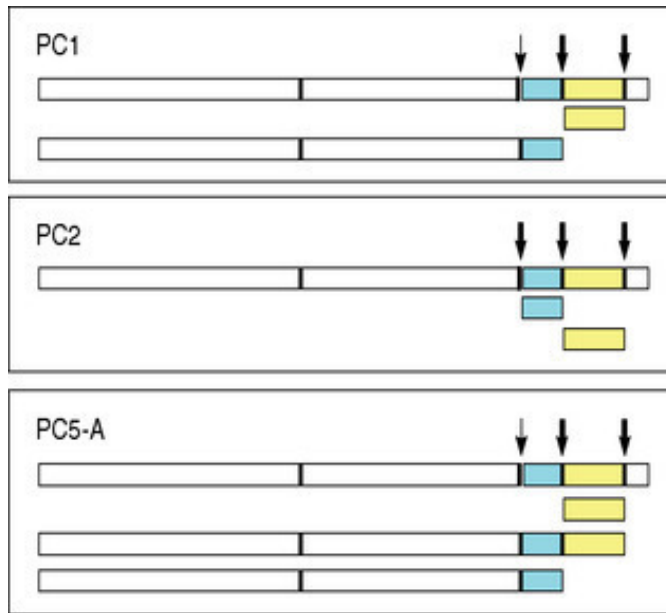


Figure 2. Cleavage specificity of pro-NT/NN and major products generated by proprotein convertase (PC) 1, PC2, and PC5-A. The same representation as in [Figure 1](#) is used. Arrow sizes are indicative of the extent to which each dibasic is cleaved by the PCs.

Several pieces of evidence indicate that PC2 is indeed the major brain pro-NT/NN convertase. Thus, PC2 is the most abundant prohormone convertase in the brain and therefore, not unexpectedly, co-localizes extensively with immunoreactive NT in neuronal populations that express pro-NT/NN. It is interesting that subsets of NT/PC2-immunopositive neurons are also positive for PC1 and PC5-A, but in general, those neurons expressing PC1 do not contain PC5-A and vice versa. PC2 plays a major role in processing brain pro-NT/NN, which is further demonstrated in PC2^{-/-} mice in which brain levels of NN are decreased by more than 50% with a compensatory increase in the levels of large NN. This indicates that processing at site 2 is impaired, as would be expected from the cleavage specificity of PC2. There is also a 20% reduction in NT levels in PC2^{-/-} mice, which is less than would be expected, had PC2 been the sole pro-NT/NN convertase in brain, supporting the idea that PC1 and PC5-A are also active in processing brain pro-NT/NN.

Degradation

Whereas classical neurotransmitter action in the synapse is often ended through specific reuptake of the transmitter into nerve terminals, the favored mechanism for terminating neuropeptide action entails proteolytic degradation. NT degradation was initially studied *in vitro* with brain membranes, which identified primary cleavage sites in the NT sequence: Arg⁸-Arg⁹, Pro¹⁰-Tyr¹¹, and Tyr¹¹-Ile¹² peptide bonds. The use of general and specific peptidase inhibitors showed that the Arg⁸-Arg⁹ cleavage was due to endopeptidase EC 3.4.24.15 (also known as thimet oligopeptidase) and that EC 3.4.24.11 (also referred to as enkephalinase, neutral endopeptidase, neprilysin, CD10, or CALLA) was entirely responsible for the Tyr¹¹-Ile¹² cleavage and only partly responsible for that at the Pro¹⁰-Tyr¹¹ bond. Finally, an unidentified metallopeptidase was shown to account for the remainder of the Pro¹⁰-Tyr¹¹ bond-cleaving activity. This enzyme, endopeptidase EC 3.4.24.16 (also designated neurolysin), was later purified and cloned. These data are summarized in [Figure 3](#). All three peptidases belong to the class of Zn-metalloendopeptidases and are specialized in cleaving small bioactive peptides in the extracellular space. They have a wide distribution in the brain and peripheral tissues, and it was indeed demonstrated in all the tissues examined that NT was degraded by a combination of the three metallopeptidases. Their action on NT effectively leads to complete biological inactivation of the peptide as its ability to activate its receptors is entirely borne by the C-terminal hexapeptide sequence NT(8-13) which is precisely the target of the metalloendopeptidases. Other peptidases are involved in further degradation of the NT breakdown products generated by the three metallopeptidases ([Figure 3](#)). They do not act as primary inactivating enzymes and are therefore less relevant to NT inactivation.

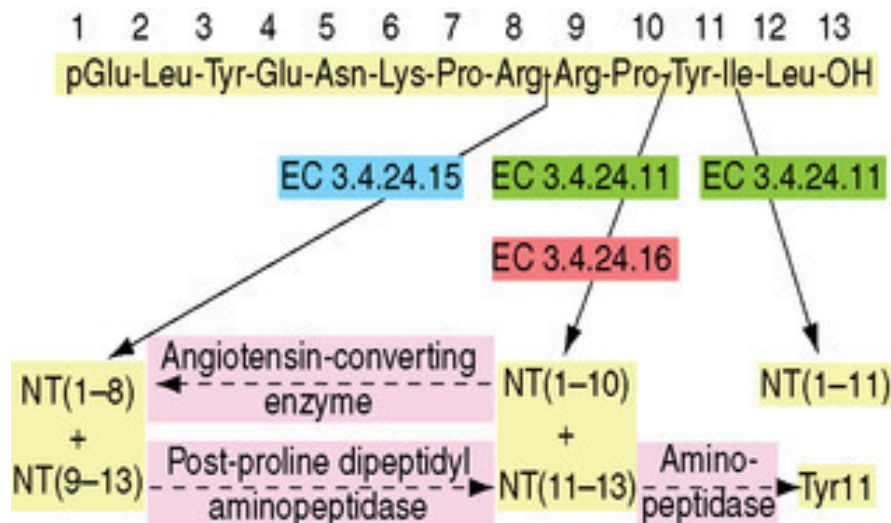


Figure 3. Scheme of NT degradation in brain. Primary inactivating cleavages are indicated by solid arrows, and further degradation of breakdown products by dashed arrows. The primary inactivating metallopeptidases, EC 3.4.24.11, 15, and 16, are highlighted in green, blue, and red, respectively. The secondary cleaving enzymes are boxed in light violet.

Brain Distribution of NT

Originally isolated from bovine hypothalamus, the neuropeptide NT was further localized throughout the central nervous system of several mammalian species, such as rat, calf, monkey, and human. Initial radioimmunoassay, carried out on rat brain extracts, revealed that the highest concentrations of NT immunoreactivity are detected in the hypothalamus, amygdala, spinal cord, and brain stem structures such as periaqueductal gray, dorsal raphe nucleus, substantia nigra, and ventral tegmental area (VTA). Subsequently, high densities of NT-immunoreactive nerve cell bodies and processes were detected throughout the brain and spinal cord by immunohistochemistry ([Figure 4](#)). In particular, NT-containing axonal fibers and/or NT-positive cell bodies are abundant in the nucleus accumbens; bed nucleus of the stria terminalis; medial and lateral septal nuclei; central, basolateral, and medial amygdaloid nuclei; subparafascicular and gustatory nuclei of the thalamus; paraventricular, and arcuate hypothalamic nuclei; lateral hypothalamus; substantia nigra; VTA; periaqueductal gray; raphe nuclei; locus coeruleus; nucleus of the solitary tract; and substantia gelatinosa of the spinal trigeminal nucleus and dorsal horn of the spinal cord. The topographic distribution of NT immunoreactivity overlapped extensively with that of NT-expressing cells as determined by *in situ* hybridization histochemistry. There were a few regions, however, in which NT immunolabeling appeared more restricted than from *in situ* hybridization data. For instance, the subiculum and CA1 region of the hippocampal formation, which were reported to contain high levels of NT messenger RNA (mRNA), were barely immunoreactive. These discrepancies could be due to selective targeting of NT peptides to neuronal processes.

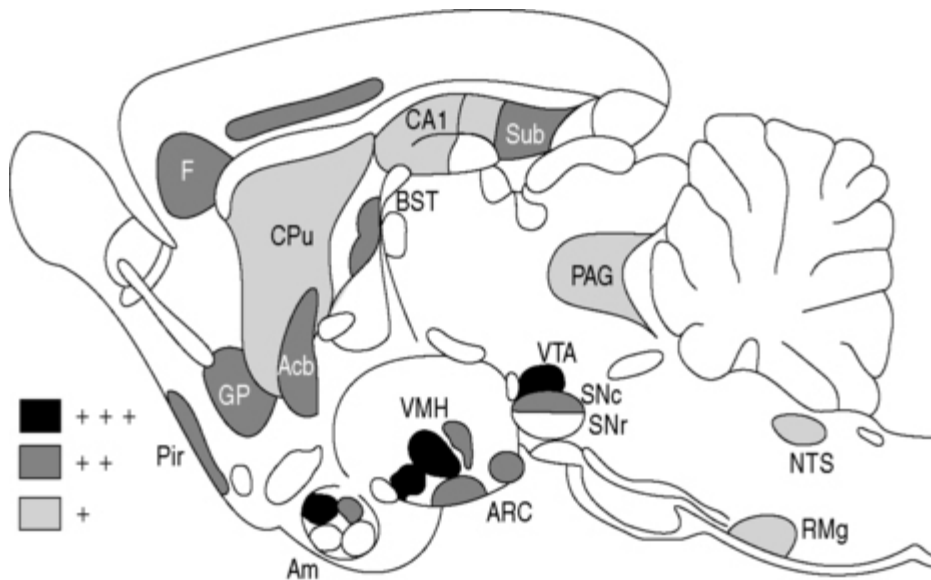


Figure 4. Topographic distribution of NT-containing neurons in the rat central nervous system. Schematic mid-sagittal section of the rat brain provides an overview of the distribution of NT immunoreactivity throughout the neuraxis. Acb, accumbens nucleus; Am, amygdaloid nucleus; ARC, arcuate hypothalamic nucleus; BST, bed nucleus of the stria terminalis; CA1, field CA1 of Ammon's horn; CPu, caudate-putamen; F, frontal cortex; GP, globus pallidus; NTS, nucleus of the solitary tract; PAG, periaqueductal gray; Pir, piriform cortex; RMg, raphe magnus; SNc, substantia nigra, compacta part; SNr, substantia nigra, reticulata part; Sub, subiculum; VMH, ventromedial hypothalamic nucleus; VTA, ventral tegmental area.

In the developing rat brain, NT was found, by radioimmunoassay, immunohistochemistry, and *in situ* hybridization, to be expressed very abundantly during the first two postnatal weeks, after which its concentration rapidly decreased to adult levels. This overexpression is regionally selective, affecting predominantly cells located in the retrosplenial and cingulate cortices, the pyramidal cell layer of the subiculum and hippocampal CA1, and the mitral cell layer of the olfactory bulb. The transient overexpression of NT during brain development suggests that this neuropeptide may be involved in the establishment of neuronal circuitry.

NT Agonists and Antagonists

Peptidic compounds

As for any other peptide, the development of potent and selective NT agonists and antagonists is critical for defining the interaction of NT with its receptors, investigating

the role of the various receptor sub-types in mediating the central actions of NT and, in the long run, developing novel therapeutic avenues. Structure-activity relationship (SAR) studies including a gradual truncation of the peptide sequence demonstrated that the C-terminal fragment NT(8-13) (Arg⁸-Arg⁹-Pro¹⁰-Tyr¹¹-Ile¹²-Leu¹³) is sufficient for receptor binding and ligand efficacy. However, as indicated in **Figure 3**, this C-terminal hexapeptide NT(8-13) exhibits a very short *in vivo* half-life (< 2 min), due to the cleavage of three of the five peptide bonds of this fragment by different endo- and exo-peptidases.

Thereby, several strategies have been developed over the last three decades to overcome the physicochemical limitations of the NT peptide. Notably, successful approaches have been applied to increase their chemical stability, reduce their rapid clearance and enzymatic degradation, and finally improve their ability to achieve optimal CNS concentration by crossing the blood-brain-barrier (BBB). Most of these compounds are highly modified versions of NT(8-13), with reduced peptide bonds, N-terminal methylation, non-natural amino acid substitution, backbone modification, cyclization, and/or other structural changes that increase stability of the molecule in the blood. Over the years, SAR studies have provided valuable insight into the role of each amino acid in this hexapeptide, as summarized in **Figure 5**.

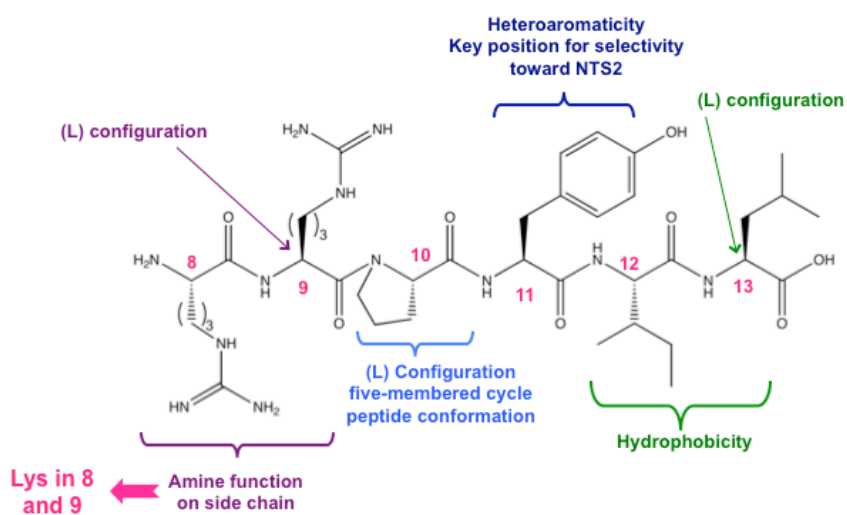


Figure 5. Structure-activity relationship (SAR) of the C-terminal active fragment NT(8-13).

A series of pseudopeptide NT analogs was produced by systematically replacing the five peptide bonds in NT(8-13) with hydrolytically stable CH₂NH (reduced) bonds, which are not cleaved by peptidases. All of these analogs were synthesized with a free amino terminus (H derivatives) or with an N-terminal *tert*-butyloxycarbonyl group (BOC

derivatives) to limit the action of aminopeptidases. Some of these NT analogs were indeed found to produce potent and long-lasting NT-like effects after *in vivo* administration. Introduction of non-natural amino acids is another alternative to gain stability, but such modification might result in NT analogs with different selectivity towards NT receptors and distinct pharmacological profiles. Detailed SAR studies have revealed that the presence of basic residues with positively charged side-chains at positions 8 and 9 is of critical importance for high-affinity receptor binding and peptide activity. Instead of Arg, Lys is often used in position 8, 9 or both, since protection of amine side chains are easier to handle in peptide synthesis. Position 8 can also accommodate N α -methylation and D-configuration providing resistance to exoprotease cleavage. As already mentioned, the pseudo-peptide analog with a reduced bond between those two Lys residues, JMV 449, is a full agonist, and analogs deriving from this lead compound are resistant to the endopeptidase 24-15 and most of them retain affinity and activity, resulting in more stable compounds. The modification of proline in position 10 has been extensively studied considering the importance of this central residue for peptide conformation. Basically, replacement of proline with aromatic derivatives and cycle extension or reduction results in loss of affinity. However, 4-substituted proline surrogates are well tolerated, especially when the substitution triggers an *exo*-puckered conformation to the pyrrolidine ring. In general, the Pro¹⁰ position seems more tolerant of substitution by amino acids that favor a reverse turn, rather than those favoring an extended conformation. Stronger conformational constraints as beta-turn mimics or spiro lactam residues have been proposed to explore the bioactive conformation of NT(8-13). The importance of the Tyr residue in position 11 of the NT molecule and the orientation of a steric bulk in this position were also investigated. These studies indicated that the presence of aromatic residues in position 11 is essential for receptor binding and selectivity. However, the opposite configuration (D-Tyr and D-Trp) is not detrimental to the activity, contrary to the suppression of the heteroatom. Furthermore, extension of aromaticity and changes in side-chain orientation seem to favor NTS2 selectivity. Indeed, high NTS2 selectivity has been observed with a peptide-peptoid hybrid resulting from replacement of Tyr with N-homotyramine, also providing resistance to proteolytic degradation. Finally, hydrophobicity of the C-terminal end is essential for receptor binding. Replacement of Ile or/and Leu with more hydrophobic non-natural amino acids, such as *tert*-leucine in many NT analogs or trimethylsilylalanine in JMV 2007 (H-Lys-Lys-Pro-Tyr-Ile-TMSAla-OH), results in both improved affinity and better metabolic stability. However, changing the L-configuration in position 13 and introducing beta-amino acid to prevent exoprotease cleavage induce an important loss in receptor binding.

Combination of several modifications has led to the development of metabolically stable analogs. In particular, charge suppression at the N- and C-terminal extremities seems

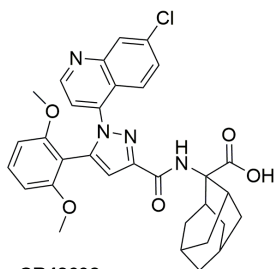
to improve BBB crossing. The first two BBB-crossing NT(8–13) analogs, NT-1 and NT-2 (also referred to as Eisai-1 and Eisai-2 compounds), were modified at amino acid residues 8, 9, 11, and 12. Specifically, N-methyl-Arg, Lys, Trp and *tert*Leu were substituted for Arg, Arg, Tyr and Ile, respectively. These analogs cause the same behavioral changes as centrally injected NT(8-13) when administered *in vivo*, including hypothermia and antinociception. Likewise, PD149163, a reduced amide NT(8-13) exhibiting selectivity for NTS1 (H-Lys-Y[CH₂NH]-Lys-Pro-Trp-*tert*Leu-Leu-OEt) showed improved metabolic stability after systemic administration and maintained the *in vivo* activities of the native NT peptide. Subsequently, several additional systemically infused but centrally acting analogs were synthesized by combining N-terminal modifications and incorporation of non-natural amino acids at position 8, 9, 11, 12 and 13. Notably, this series of hexapeptide analogs of NT (8-13) includes NT69L (H-NMe-Arg-Lys-Pro-*neo*Trp-*tert*Leu-Leu-OH), JMV 1842 (H-NMe-Arg-Lys-Pro-Trp-Ile-*tert*Leu-OH) and ABS212 (H-N^ωMe₃-αMe-Orn-Arg-Pro-Tyr-*tert*Leu-Leu-OH) that cross the BBB very efficiently and exhibit strong agonistic *in vivo* activity. Finally, cyclisation of the NT(8-13) led to compounds JMV 1193 (c(Lys-Lys-Pro-Tyr-Ile-Leu)) and JMV 2012, a cyclic dimer (c(Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu)), both showing a good bioavailability after i.v. or oral administration.

Non-peptidic compounds

A better understanding of the central effects of endogenous NT has also been provided by the development of potent and selective antagonists. To this end, a number of non-peptide antagonists were developed, including Pfizer's UK-73,093, Merck's L-734836, and Parke-Davis' PD-156425. However, the most potent and selective non-peptide NT antagonist developed so far is Sanofi's SR48692 (also called meclintant or remintant) ([Table 1](#)). This compound is orally active, crosses the BBB, and has a long-lasting action. It also displays a much higher affinity for NTS1 (IC₅₀ = 5.6 nM) than for NTS2 (IC₅₀ = 300 nM) and NTS3 (IC₅₀ > 1 μM), which makes it a good tool for differentiating the contribution of NTS1 from that of other NT receptors to the central effects of NT. Given intra-peritoneally or orally, SR48692 suppresses the turning behavior induced by unilateral injection of NT in mouse neostriatum. It also antagonizes the locomotor activity evoked by NT injection into the ventral tegmental area. The adamantyl group of SR48692 has been replaced by a cyclohexyl group differently branched resulting in the two stereoisomers of S configuration (SR48527) and R configuration (SR49711) with the same and 100-fold less potency, respectively. A follow-up compound, SR142948A, was then introduced which is more soluble but non selective in that it recognizes both NTS1 and NTS2 receptors with high affinity (IC₅₀ = 1-4 nM). This antagonist blocks both hypothermic and analgesic effects induced by centrally administered NT.

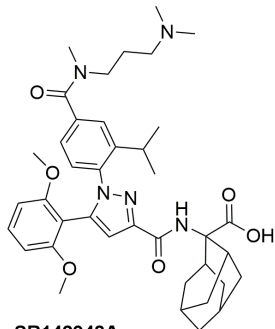
A virtual screening using a shape-based superimposition method (ROCS, Rapid Overlay of Chemical Structures) from the structure of SR48527 has allowed the identification of two hits with partial agonist activity and potency in the moderate micromolar range. One of these compounds issued from Wyeth research ($EC_{50} = 178 \mu\text{M}$, $E_{\text{max}} = 17\%$) was further optimized to lead to SR-12062 showing a full efficacy profile ($EC_{50} = 2 \mu\text{M}$, $E_{\text{max}} = 100\%$). A non-high throughput screening (HTS) scaffold-hop program identified the probe candidate ML301 and associated analogs ([Table 1](#)). This compound exhibited full agonist behavior (79 – 93%) with an half maximal effective concentration (EC_{50}) of 2.0 – 4.1 μM against NTS1 in the primary assay and 93% efficacy at 298 nM in the Ca^{2+} mobilization assay (FLIPR) with a good selectivity relative to NTS2. In a further medicinal chemistry optimization, a quinazoline-based series led to the discovery of ML314, a brain penetrant agonist that exhibited full agonist behavior (100 %) on NTR1 ($EC_{50} = 1.9 \mu\text{M}$) with a good selectivity against NTS2. ML314 was not able to stimulate Ca^{2+} mobilization, but showed a β -arrestin biased agonist behavior.

While imidazole-derivatives investigation provides NTS1 selective agonists, such as ML301, indol- and pyrazol-derivatives afford selective NTS2 compounds, such as NTRC-824 and NTCR-739, respectively ([Table 1](#)). The compound NTRC-824, structurally related to the Wyeth compound, is selective for NTS2 versus NTS1 and exhibits antagonist properties in the NTS2 FLIPR assay. NTRC-739, mixing both structures of SR142948A and SR48527 with a cyclohexyl group differently branched, behaves as a potent partial NTS2-selective agonist in regard to the FLIPR assay.



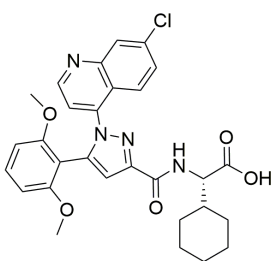
SR48692

NTS1 IC₅₀ = 5.6 nM
NTS2 IC₅₀ = 300 nM



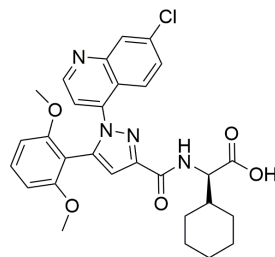
SR142948A

NTS1 & NTS2 IC₅₀ = 1-4 nM



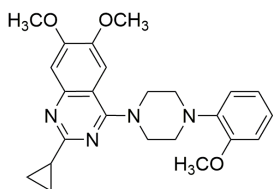
SR48527

NTS1 IC₅₀ = 85 nM



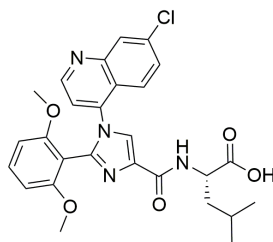
SR49711

NTS1 IC₅₀ = 6200 nM



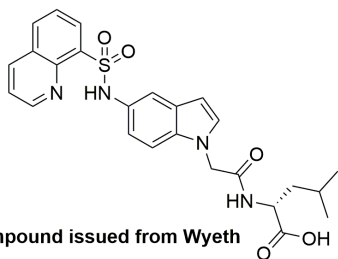
ML314

NTS1 EC₅₀ = 2 μM (100%)
NTS2 EC₅₀ > 80 μM



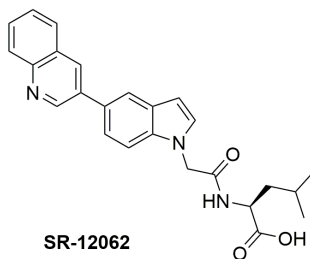
ML301

NTS1 EC₅₀ = 298 nM (93%)
NTS2 EC₅₀ > 80 μM



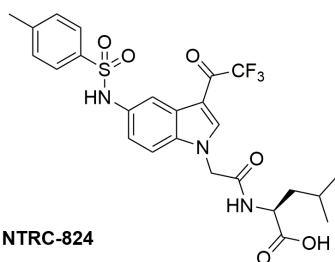
Compound issued from Wyeth

NTS1 EC₅₀ = 178 μM (17%)



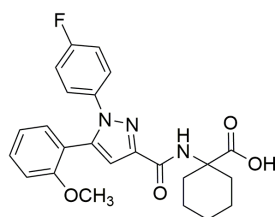
SR-12062

NTS1 EC₅₀ = 2 μM (100%)



NTRC-824

NTS1 IC₅₀ > 30 μM
NTS2 IC₅₀ = 202 nM



NTRC-739

NTS1 IC₅₀ > 25 μM
NTS2 IC₅₀ = 153 nM

Table 1. Structure of non peptidic agonists and antagonists.

NT Receptors and Signaling

Central effects of NT and its analogs are mediated by the activation of at least three different receptor subtypes, identified as NTS1, NTS2, and NTS3 (also called gp95/sortilin). A summary of the biochemical, biological, and pharmacological properties of these receptors is provided in [Table 2](#).

<i>NT receptor subtypes</i>	<i>NTS1 (NTRH)</i>	<i>NTS2 (NTRL)</i>	<i>NTS3 (gp95/sortilin)</i>
Receptor classification	G-protein-coupled receptor (7 TM)	G-protein-coupled receptor (7 TM)	Type I receptor (1 TM)
Cloned	rNTS1	rNTS2	rNTS3
	mNTS1	mNTS2	mNTS3
	hNTS1	hNTS2	hNTS3
Receptor size	rNTS1 and mNTS1 = 424 aa	rNTS2 and mNTS2 = 416 aa	mNTS3 = 825 aa
	hNTS1 = 418 aa	Variant isoform (281 aa, 5 TM)	hNTS3 = 833 aa
		hNTS2 = 410 aa	
Molecular weight	50 and 60 kDa	45 kDa	100 kDa
Gene characteristics	hNTS1 gene localized to the long arm (20q13) of chromosome 20	mNTS2 gene mapped at 6cM from the centromere on chromosome 12	hNTS3 gene mapped to the proximal short arm of chromosome 1
	Tetranucleotide repeat polymorphism located <3 kb from the gene	Two splice variants derived from a single gene	Two sortilin mRNA transcripts 3.5 and 8.0 kb expressed in human CNS
$K_{dv} = 0.1-0.3 \text{ nmol l}^{-1}$ (rat brain synaptic membranes)	$K_{dv} = 0.1-0.3 \text{ nmol l}^{-1}$	$K_{dv} = 2-4 \text{ nmol l}^{-1}$	$K_{dv} = 0.3 \text{ nmol l}^{-1}$ (CHAPS-solubilized human brain)

<i>NT receptor subtypes</i>	<i>NTS1 (NTRH)</i>			<i>NTS2 (NTRL)</i>				<i>NTS3 (gp95/sortilin) receptors</i>
								$K_{dv} = 17 \text{ nmol l}^{-1}$ (CHAPS-solubilized cloned hNTS3 in COS-7 transfected c□)
Maximal binding capacity (Bmax) (adult rat brain homogenate)	Bmax = 13 fmol/mg			Bmax = 170 fmol/mg				Bmax = 150 fmol/mg (CHAPS solubilized human brain receptors)
Levocabastine sensitivity	-			+				-
GTP sensitivity	+			-				?
Na ⁺ sensitivity	IC50 = 15 mmol l ⁻¹ (Asp-113)			IC50 = 200 mmol l ⁻¹				?
Internalization	65–70% of total binding			60% of total binding				75% of total binding
	$t_{1/2} = 7 \text{ min}$			$t_{1/2} = 10 \text{ min}$				$t_{1/2} = 5 \text{ min}$
	C-terminal tail (TLY motif)			Intracellular loop 3 (I3)				?
Recycling	-			+				+
				Y237				
Cellular localization	Mainly neurons			Neurons and astrocytes				Neurons and glia
G-protein coupling	Gi/o, Gs, Gq, G ₁₃ and β-arrestins			ND				ND
Signal transduction		(+)	(-)		(+)	(-)	(*)	
Human All systems	Phospholipase C	NT	SR48692	Phospholipase C	NT	NT	NT	Modulation of NTS1-mediated IP turnover and MAPKs

<i>NT receptor subtypes</i>	<i>NTS1 (NTRH)</i>			<i>NTS2 (NTRL)</i>			<i>NTS3 (gp95/sortilin)</i>	
	↑ cytosolic [Ca ²⁺]	NN	SR142948A	↑ cytosolic [Ca ²⁺]	Levo	Levo	Levo	
	↑ IP			↑ IP	SR48692		JMV 431	Desensitization of calcium mobilizing effects of NT
	Stimulation of cGMP, cAMP			MAPK activation	SR142948A			
	MAPK activation			Arachidonic acid release				Induction of PI3 kinases and MAPK pathways
	Krox-24 gene induction			Constitutive activity				
				↑ IP				
Rat								
All systems	Inhibition of cAMP production	NT	SR48692	↑ cytosolic [Ca ²⁺]	JMV449			
		NN	SR142948A	MAPK activation	NT			
	Arachidonic acid production				SR48692			
					Levo			
Primary cultured cerebellar granule				↑ cytosolic [Ca ²⁺]	SR48692			
				MAPK activation	NT			
					Levo			
Mouse All systems				Activation of Ca ²⁺	NT			Activation of Akt kinases and ERK1/2
				dependant Cl ⁻	Levo			
				conductance	SR48692			
Physiological implications	NT-induced turning behavior							Elicits migration of

<i>NT receptor subtypes</i>	<i>NTS1 (NTRH)</i>	<i>NTS2 (NTRL)</i>	<i>NTS3 (gp95/sortilin)</i>
			glial .
	Modulation of dopamine transmission	NT-induced analgesia	NT-induced c□ growth
	NT-induced growth	Effects of NT not blocked by SR48692	NT-induced c□ growth blocked by SR48692 Sorting and internalization of NT to the TGN
	NT-induced hypothermia	Effects of NT blocked by SR142948A	Inhibitory effect of NT on the pro-apoptotic effect of NTS3 and p75NTR interaction
	Effects of NT blocked by SR48692 and SR142948A		

Table 2. Biochemical, pharmacological, and physiological properties of NT receptor subtypes NTS1, NTS2, and NTS3.

aa, amino acids; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; ERK, extracellular signal-regulated kinase; h, human; K_{av} , dissociation constant; m, mouse; MAPK, mitogen-activated protein kinase; ND, nondefined; NN, neuromedin neurotensin; NT, neurotensin; TGN, *trans*-Golgi network; TM, transmembrane domains; ., cells; +, agonist; -, antagonist; *, inverse agonist; Levo, Levocabastine; ↑, increase

NTS1 and NTS2 belong to the G-protein-coupled receptor family and were initially distinguished pharmacologically on the basis of their affinities for NT and their differential sensitivity to the antihistamine-1 drug levocabastine. Referred to as the high-affinity ($K_{av} = 0.1-0.3 \text{ nmol l}^{-1}$) levocabastine-insensitive binding site, NTS1 has been linked, through Gi/o, Gq, Gs and G₁₃-protein coupling, to a variety of signaling cascades, including formation of cyclic guanosine monophosphate and cAMP, phospholipase C, and inositol phosphate signaling cascades; arachidonic acid production; and activation/inhibition of mitogen-activated protein kinases (ERK1/2 and JNK) and serine/threonine protein kinase Akt. Association of NT to NTS1 induces

internalization of receptor-ligand complexes into NTS1-expressing cells via clathrin-coated pits. The endocytic process is dependent on the integrity of the C-terminal extremity and requires the recruitment of both isoforms of β -arrestin 1 and 2, dynamin and intersectin. NTS1 internalization is accompanied by NT endocytosis. However, the intracellular trafficking patterns of NTS1 and of NT may be distinct, depending on the time period of agonist exposure. Biochemical and cell imaging studies revealed that under acute agonist exposure, internalized NTS1 receptors are targeted to lysosomes for degradation whereas under prolonged agonist exposure, NTS1 is routed toward the perinuclear *trans*-Golgi recycling compartment. NTS1 gene activation and *de novo* receptor synthesis therefore contribute to cell resensitization.

The levocabastine-sensitive, low-affinity NT receptor (NTS2) was cloned from mouse, rat, and human brain by a strategy based on sequence homology with the known NTS1 receptor. NTS2 shares approximately 60% amino-acid homology with NTS1 and binds NT and levocabastine with similar affinity ($K_{dv} = 2\text{-}10 \text{ nmol l}^{-1}$). In contrast to NTS1, the signaling properties of NTS2 are still controversial, exhibiting cell-type expression- and species-dependent pharmacological properties ([Table 2](#)). For instance, depending on the heterologous expression cell system (oocytes, CHO, COS-7, and HEK293 cells) used to characterize the signal transduction mechanisms of the NTS2, levocabastine and NT analogs were reported to act as agonists, inverse agonists, or competitive antagonists at NTS2 sites. The most convincing data supporting an agonist role for endogenous NT on NTS2 receptors are, however, the ability of NT to induce a sustained mitogen-activated protein kinase (MAPK) p42/p44 activation in rat cerebellar granule cells expressing endogenous NTS2. Different results have also been reported on the constitutive activity of the human NTS2 (hNTS2) transfected in COS and CHO cells.

Although the agonistic properties of NT on the NTS2 receptor remain a debate, NTS2 receptors were found to efficiently internalize on NT binding via a clathrin-coated pit and dynamin-dependent mechanism, a property usually associated with an activation by agonists. Note that ligand-receptor complexes were found to form clusters at the cell surface but not to internalize in cultured astrocytes following NT stimulation, suggesting that NTS2 internalization capacities are likely cell-type dependent. In contrast to NTS1, sequestered mouse NTS2 (mNTS2) receptors are efficiently recycled to the plasma membrane. The third intracellular loop and in particular the tyrosine-237 residue are crucial for mNTS2 receptor internalization and recycling, respectively. Recently, the neuron-enriched endosomal protein of 21 kDa (NEEP21) was shown to be involved in mNTS2 recycling. However, species discrepancies are here again detected. In opposition to mNTS2, the hNTS2, in which a cysteine residue naturally replaces the tyrosine in position 237, does not undergo recycling following endocytosis. The underlying mechanisms responsible for NTS2 receptor preservation at the cell surface seem to be different depending on the length of agonist exposure. Indeed, whereas

under acute agonist exposure, maintenance of cell surface receptors seems to be related to NTS2 recycling, under persistent exposure to NT, this preservation of cell plasma membrane NTS2 receptor densities appears to involve recruitment of spare receptors from internal stores, thereby preventing functional desensitization. Originally identified as the intracellular sorting protein sortilin, the NTS3 receptor is unique among neuropeptide receptors in that it is a single transmembrane domain protein. NTS3 exhibits striking sequence similarities with proteins involved in intracellular transport and trafficking. Accordingly, several studies have demonstrated a predominant association of NTS3 with intracellular sorting organelles such as the Golgi apparatus, endosomes, and Glut4 vesicles. Although only 5-10% of this receptor is expressed at the cell surface, NTS3 efficiently internalizes NT. NTS3 has the ability to bind several ligands in addition to NT, such as the lipoprotein lipase, the endoplasmic reticulum resident receptor-associated protein, and its own propeptide released after maturation of the precursor form by furin, suggesting that its role probably exceeds that of an NT signaling receptor. Binding of NT to NTS3 was reported to elicit proliferative responses in a variety of cancer cell lines and to alter agonist-induced cellular trafficking and signaling of NTS1 in HT29 human colon cancer cells. However, the strongest evidence that NTS3 participates in NT signaling comes from the demonstration that NTS3 mediates the NT-induced migration of human microglial cells by a mechanism dependent on the stimulation of both MAPK and phosphoinositide-3 kinase pathways. Recently, sortilin has also been shown to interact with the p75 neurotrophin receptor (p75NTR) in the induction of apoptosis on binding unprocessed nerve growth factor (NGF) proNGF. However, the occupancy of NTS3 by NT prevents the proNGF-induced formation of the sortilin-p75NTR receptor complex and thereby counteracts the p75NTR-induced neuronal cell death. Taken together, these studies suggest that NT binding to NTS3/sortilin may play an important role in inflammation and neurotoxicity.

Localization of NT Receptor Subtypes

Initial evidence for the existence of NT receptors was derived from radioligand-binding experiments on membranes prepared from brain and gastrointestinal tissues. However, it is only with the application of autoradiographic-binding techniques, using [³H]NT or [¹²⁵I]NT as radioligands, that a comprehensive picture of brain NT receptor distribution began to emerge. Subsequently, the cloning of NT receptors allowed the generation of mRNA probes and antibodies to investigate their distribution by Northern blotting, *in situ* hybridization, and immunohistochemistry at light and electron microscopic levels.

NTS1 Receptors

Autoradiographic studies using [¹²⁵I]NT in the presence of blocking concentrations of levocabastine (to prevent labeling of the low-affinity site) have revealed a selective association of NTS1-binding sites with selective neuronal populations throughout the hypothalamus, basal forebrain, and limbic system (Figure 6). High concentrations of NTS1-binding sites were distributed in the perikarya and dendrites of subsets of chemo-specific neurons in a variety of brain regions, including DA neurons in the ventral midbrain, cholinergic neurons in the basal forebrain, and vasoactive intestinal peptidergic neurons in the suprachiasmatic nucleus of the hypothalamus. This distribution is consistent with reported NTS1-mediated NT effects on locomotion, memory, cognition, circadian rhythms, and sleep-waking cycle, as well as on the regulation of hypothalamo-pituitary functions. Accordingly, all these regions were later found to contain numerous nerve cell bodies expressing NTS1 mRNA and/or NTS1 receptor proteins by *in situ* hybridization and immunohistochemistry, respectively. The highest levels of staining were observed in the islands of Calleja, diagonal band of Broca, magnocellular preoptic nucleus, pre- and para-subiculum, suprachiasmatic nucleus, anterodorsal nucleus of the thalamus, substantia nigra, and VTA.

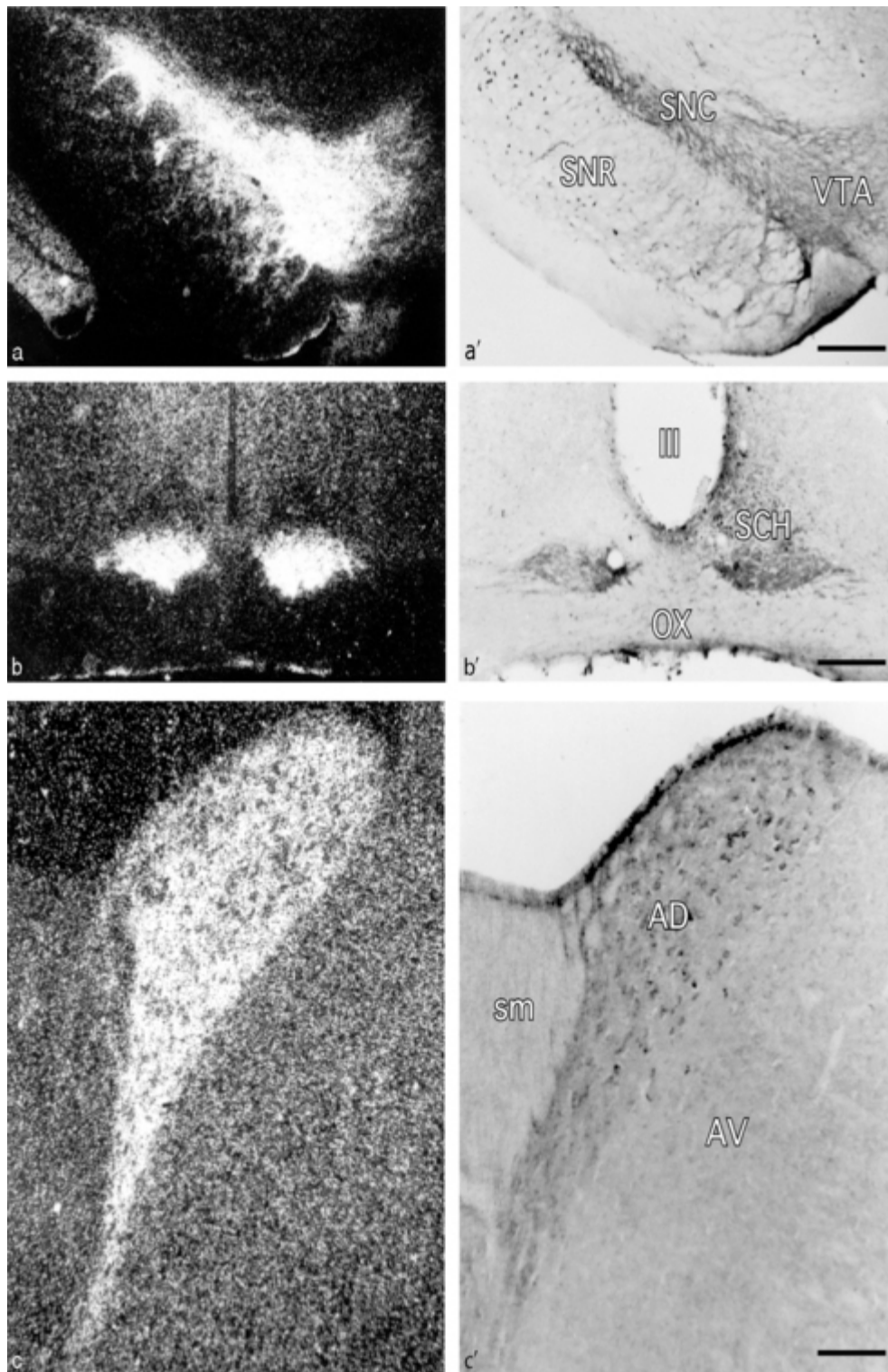


Figure 6. Comparative distribution of $[[^{125}\text{I}]]\text{NT}$ -binding sites (dark-field, left) and NTS1 immunoreactivity (bright-field, right) in the substantia nigra (SN; (a), (a')), the supraschiasmatic nucleus (SCH; (b), (b')), III: third ventricle; OX: optic chiasm and the

anterodorsal thalamic nucleus (AD; (c), (c')), sm: stria medullaris; AV: anteroventral thalamic nucleus). In all regions, the labeling patterns are strikingly similar. In the substantia nigra, compacta part (SNC) and the ventral tegmental area (VTA), NTS1 immunoreactivity is detected within both perikarya and processes, whereas in the substantia nigra, compacta reticulata (SNR), the labeling is mainly observed over dendritic arbors. Within the SCH and AD, the immunoreactivity is also apparent within perikarya and dendrites in the ventral tier of the nucleus. Scale bars = 300 μ m (a, a'); 200 μ m (b, b' , c, c'). Reprinted [Boudin H, Pélaprat D, Rostène W, and Beaudet A \(1996\)](#) Cellular distribution of neurotensin receptors in rat brain: Immunohistochemical study using an antipeptide antibody against the cloned high affinity receptor. *Journal of Comparative Neurology* 373(1): 76-89, with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Ltd.

At the cellular level, NTS1 receptors were predominantly associated with perikarya and dendrites in some regions (e.g., in the basal forebrain, ventral midbrain, pons, and rostral medulla) and with axons and axon terminals in others (e.g., in the lateral septum, bed nucleus of the stria terminalis, neostriatum, paraventricular nucleus of the thalamus, and nucleus of the solitary tract). These labeling patterns suggest that neurons expressing NTS1 receptors target these receptors to both their somatodendritic and axonal elements, providing evidence for pre- and postsynaptic effects of NT in different brain regions. At the subcellular level, in all areas examined, a significant proportion of both immunoreactive and radiolabeled NTS1 was found in association with neuronal plasma membranes. In brief, in the nucleus accumbens, NTS1 receptors were mainly associated with the plasma membrane of axon terminals, whereas they were mostly observed over the membrane of DA neurons in the substantia nigra and VTA. By contrast, dendrites showed a higher concentration of intracellular receptors, suggesting that neurons expressing NTS1 receptors maintain large receptor reserves ([Figure 7](#)). An important fraction of these intracellular receptors are associated with the Golgi apparatus and endoplasmic reticulum, indicating that newly synthesized receptor proteins are in the process of synthesis and posttranslational modifications. Electron microscopy immunocytochemistry also revealed the presence of NTS1 receptors in endosomal vesicles, suggesting that NTS1 receptors internalize on ligand stimulation.

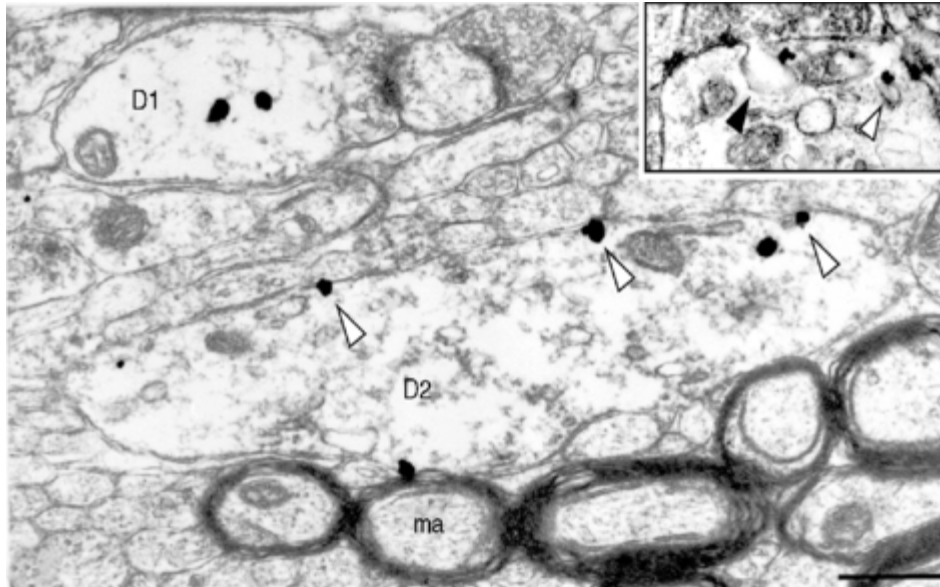


Figure 7. Immunolabeled NTS1 receptor proteins associated with dendrites (D) in rat substantia nigra. One of the labeled dendrites (D1) shows only intracellular immunogold particles whereas the other (D2) is predominantly labeled on its plasma membrane (white arrowheads). Inset: Note the presence of NTS1 proteins in association with the outer membrane of an endosomal vesicle (white arrowheads) and of an endocytic profile (black arrowheads). ma; myelinated axon. Scale bars = 0.5 μm and 0.2 μm (inset). Reproduced from [Boudin H, Pélaprat D, Rostène W, Pickel VM, and Beaudet A \(1998\)](#) Correlative ultrastructural distribution of neurotensin receptor proteins and binding sites in the rat substantia nigra. *Journal of Neuroscience* 18(20): 8473-884. Copyright (1998) by the Society for Neuroscience.

NTS2 Receptors

Early autoradiographic-binding studies, based on the displacement of specific [^3H]NT, [^{125}I]NT, or [^3H]SR142948A (a non-peptidic NT antagonist) binding by levocabastine or on the direct labeling of NTS2 receptors using [^3H]-levocabastine as radioligand, reported a widespread distribution of levocabastine-sensitive NTS2 receptor sites in adult rat brain. In conformity with the results obtained by autoradiographic approaches, *in situ* hybridization histochemistry revealed that mRNA encoding NTS2 was diffusely distributed throughout the brain. High NTS2 expression levels were detected in the olfactory system, cerebral and cerebellar cortices, hippocampal formation, and selective hypothalamic nuclei. A striking feature of NTS2 distribution was its association with every single sensory system in the brain. Moderate to dense hybridization signal and NTS2 immunoreactivity were observed in a variety of olfactory (e.g., olfactory bulb, anterior olfactory nucleus, and piriform cortex), visual (e.g., suprachiasmatic nucleus, superior colliculi, lateral geniculate body), auditory (e.g., superior olivary nuclei,

cochlear nuclei, and temporal cortex), and gustatory cerebral structures (e.g., nucleus of the solitary tract) (Figure 8). Of particular interest from a functional perspective is the intense labeling of brain stem structures involved in pain control. Indeed, NTS2 mRNA and receptor proteins were highly expressed in brain stem structures implicated in the descending control of nociceptive input, such as the periaqueductal gray, dorsal raphe nucleus, raphe magnus and pallidus, gigantocellular reticular nucleus, pars alpha, and lateral paragigantocellular nucleus. The strong NTS2 immunolabeling also found in the dorsal root ganglia and in the superficial layers of the dorsal horn of the spinal cord suggests that NTS2 receptors are involved in the modulation of primary afferent pathways and are ideally poised to regulate NT' s spinal antinociceptive actions (Figure 9).

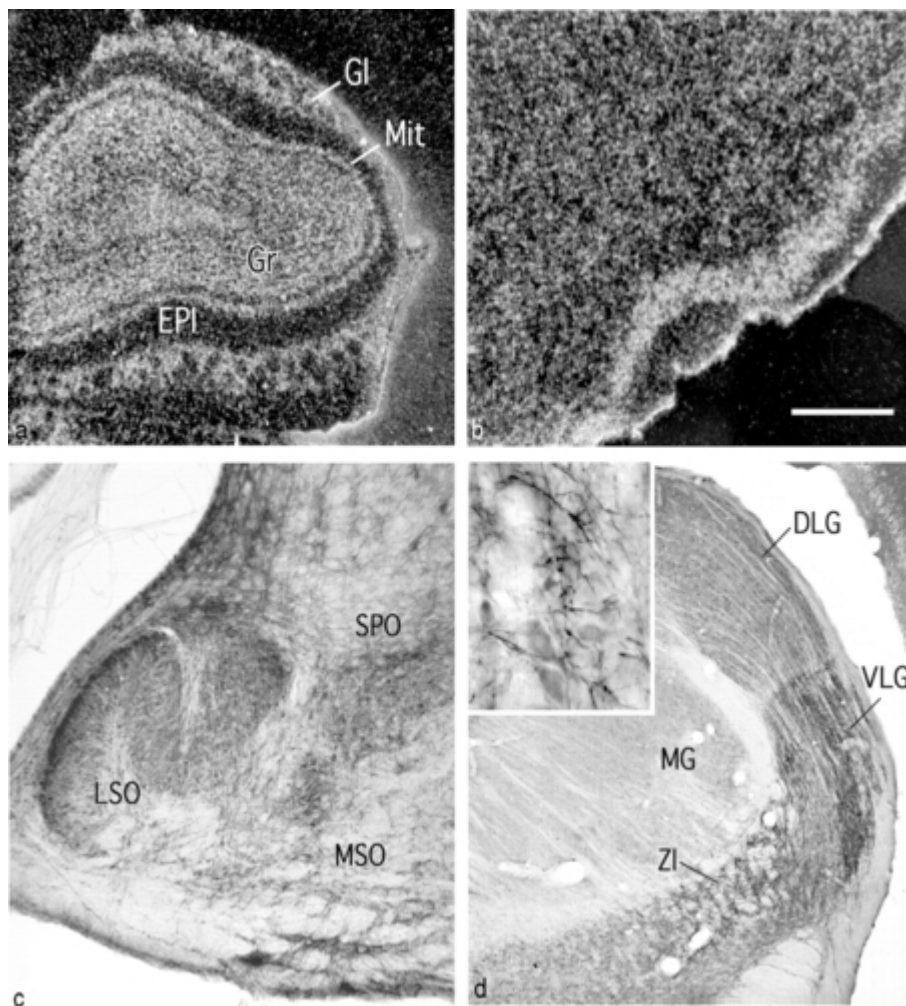


Figure 8. NTS2 receptor mRNA (a, b) and proteins (c, d), as detected by immunohistochemistry and *in situ* hybridization, respectively, in the olfactory (a, b), auditory (c), and visual (d) sensory systems. Dark-field views of sections of the olfactory

bulb (a) and piriform cortex (b). Within the olfactory bulb, intense hybridization is apparent over mitral and granular cells whereas only weak labeling is visible over plexiform and glomeruli layers. In the piriform cortex, silver grains were almost exclusively confined to layer II. Distribution of NTS2 immunoreactivity in the superior olivary complex (c) and lateral geniculate nucleus (d). In the rostral pons, the superior olivary complex exhibits a mix of labeled somata and processes. Intense labeling is observed over the dorsal and ventral tiers of the lateral geniculate nucleus as well as in the adjoining zona incerta. As shown in the inset, the NTS2 staining accounts for dendritic processes. DLG, dorsal lateral geniculate nucleus; EPI, external plexiform layer; Gl, glomeruli layer; Gr, granule cell layer; LSO, lateral superior olive; MG, medial geniculate nucleus; Mit, mitral cell layer; MSO, medial superior olive; SPO, superior paraolivary nucleus; VLG, ventral lateral geniculate nucleus; ZI, zona incerta. Scale bars = 1 mm (a, b, d); 300 μ m (c); 80 μ m inset. (a, b) Reprinted from [Sarret P, Beaudet A, Vincent JP, and Mazella J \(1998\)](#) Regional and cellular distribution of low affinity neurotensin receptor mRNA in adult and developing mouse brain. *Journal of Comparative Neurology* 394(3): 344-356, with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Ltd. (c, d) Reprinted from [Sarret P, Perron A, Stroh T, and Beaudet A \(2003\)](#) Immunohistochemical distribution of NTS2 neurotensin receptors in the rat central nervous. *Journal of Comparative Neurology*. 461(4): 520-538. with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Ltd.

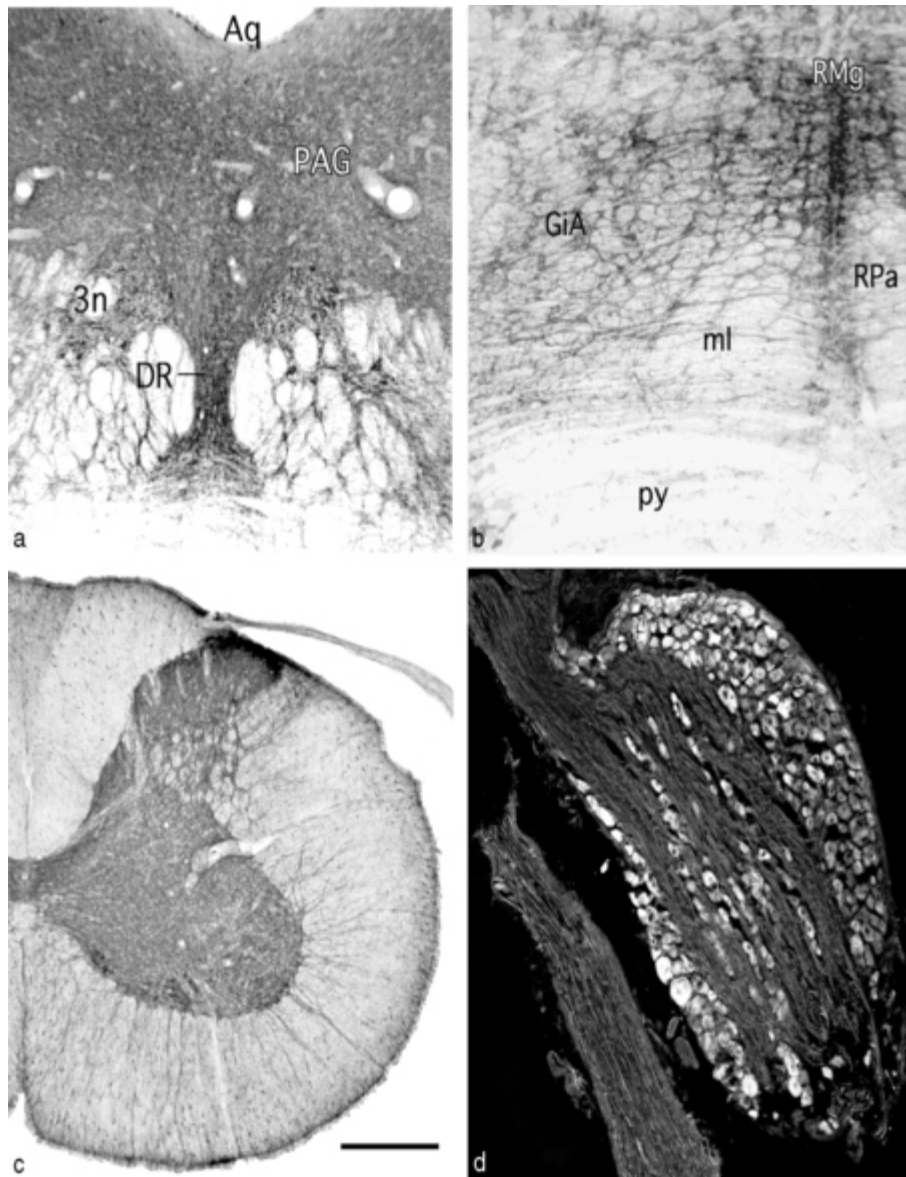


Figure 9. Distribution of levocabastine-sensitive NTS2 receptor immunoreactivity in spinal and supraspinal cerebral structures. (a) The ventral periaqueductal gray (PAG) and dorsal raphe nucleus (DR) both exhibit a dense network of immunopositive processes. (b) The gigantocellular reticular nucleus (GiA), pars alpha, exhibits numerous labeled perikarya as well as processes. Medially, the nucleus raphe magnus (RMg) is strongly stained, and the raphe pallidus (RPa) displays moderate immunoreactivity. (c) Localization of NTS2 receptors in lumbar spinal cord. Immunoperoxidase staining reveals the presence of intense NTS2 immunoreactivity within the superficial layers of the dorsal horn. A dense plexus of NTS2-immunoreactive neuronal processes is observed in laminae I and II. (d) Cellular distribution of NTS2 receptors in primary afferent neurons. Strong NTS2 labeling is evident in

subpopulations of small and large dorsal root ganglion cells. Aq, aqueduct; 3n, oculomotor nucleus; py, pyramidal tract; ml, medial lemniscus. Scale bars = 500 μ m (a), 350 μ m (b), 600 μ m (c), 200 μ m (d). (a, b) Reprinted from [Sarret P, Perron A, Stroh T, and Beaudet A \(2003\)](#) Immunohistochemical distribution NTS2 neurotension receptors in the rat central nervous system. *Journal of Comparative Neurology* 461(4): 520-536, with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Ltd. (c,d) Reprinted from [Sarret P, Esdaile MJ, Perron A, Martinez J, Stroh T, and Beaudet A \(2005\)](#) Potent spinal analgesia elicited through stimulation of NTS2 neurotensin receptors. *Journal of Neuroscience* 25(36): 8188-8196.

Biochemical, *in situ* hybridization, receptor autoradiographic, and immunohistochemical studies all concur in demonstrating that in the rodent brain, the developmental pattern of NTS2 receptors is markedly different from that reported for the NTS1s. Indeed, NTS2 receptors are absent at birth, appear late during development, and do not reach their maximal levels until adulthood. In contrast to NTS2, NTS1 is expressed before birth, peaks at around postnatal day 10, and progressively decreases thereafter, reaching adult levels between 30 and 40 days. On the basis of their ubiquitous distribution, as well as their pattern of ontogenic development, NTS2 receptors were originally surmised to be mainly associated with glial cells. Accordingly, NTS2 binding and/or mRNA were detected in association with astrocytes both *in vitro* and *in vivo*. This NTS2 glial expression was low under basal conditions but was massively up-regulated in reactive astrocytes surrounding a cortical stab wound, suggesting that NTS2 receptors may play a role in the regulation of inflammatory responses and neuronal regeneration. Despite its expression in glial cells, the cellular localization of NTS2 is predominantly neuronal. However, unlike NTS1 receptors, which were reportedly distributed over perikarya, dendrites, and axons of neurons, NTS2 receptors were predominantly, although not exclusively, associated with dendritic processes.

NTS3 Receptors

Both NTS3/sortilin mRNA and immunoreactive proteins displayed a broad distribution throughout the brain. High levels of NTS3/sortilin expression and immunoreactivity were found in neuronal cell bodies and dendrites of cortical areas such as the piriform cortex and hippocampus, the islands of Calleja, medial and lateral septal nuclei, amygdaloid nuclei, thalamic nuclei, the substantia nigra, and the Purkinje cell layer of the cerebellar cortex (**Figure 10**). Furthermore, in the brain stem, all cranial nerve motor nuclei were strongly labeled. However, even if NTS3 mRNA and immunoreactivity patterns were mainly associated with neuronal elements, NTS3 receptors were also found in association with glial cells, corresponding, in morphology, to oligodendrocytes.

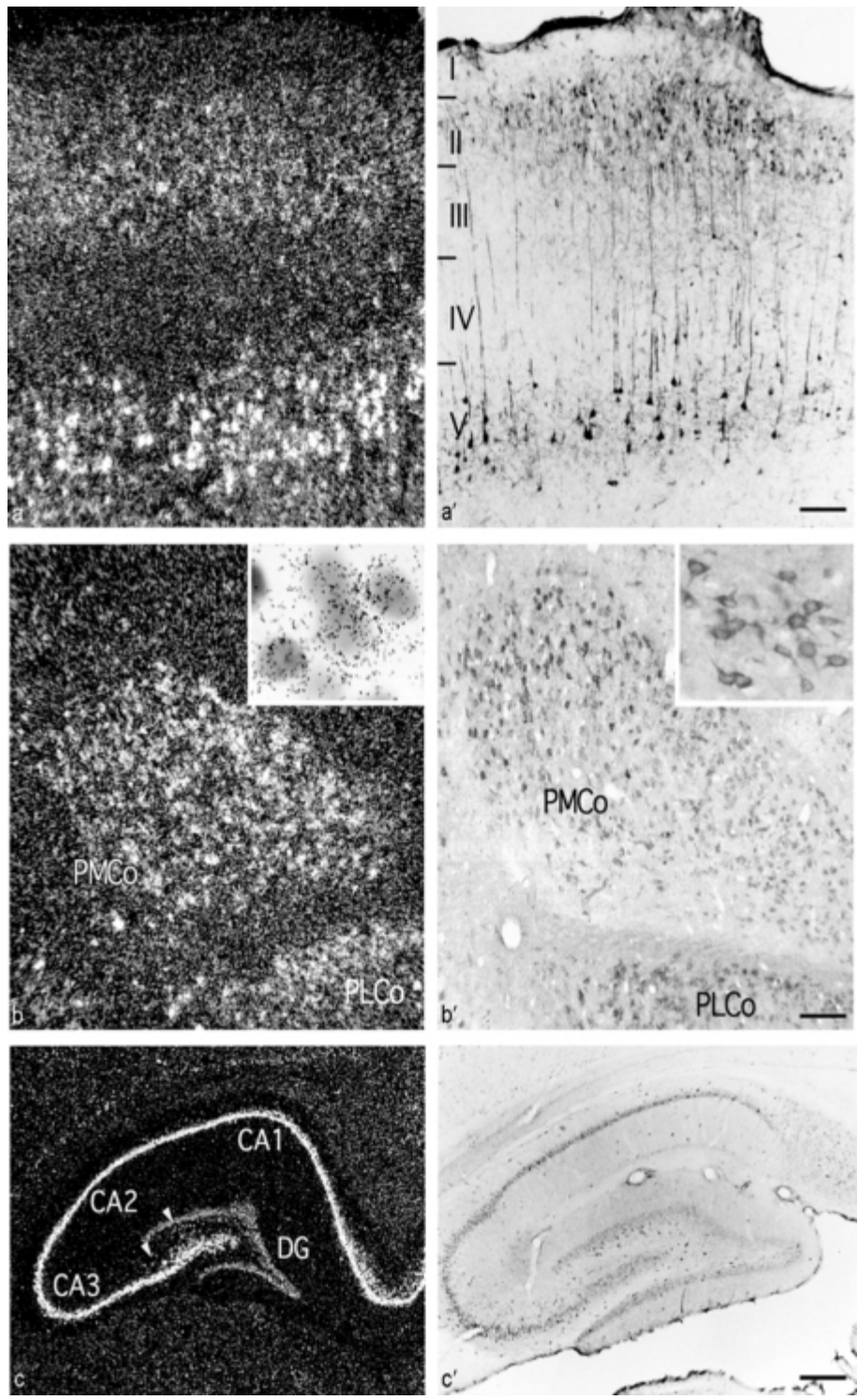


Figure 10. NTS3/sortilin *in situ* hybridization (a-c) and immunoreactivity (a' -c') in the parietal cortex (a, a'), amygdaloid complex (b, b'), and hippocampal formation (c, c'). In the parietal cortex, dark-field micrograph of NTS3/sortilin antisense probe-hybridized section and immunocytochemical analysis reveal a large number of cell

bodies in layers II/III and V. In the amygdaloid complex, sortilin immunoreactivity displays a distribution identical to the pattern of mRNA expression in both nuclei (PMCO, posteromedial cortical amygdaloid nucleus; PLCo, posterolateral cortical amygdaloid nucleus). At higher magnification (inset), the labeling is clearly over large neuronal cell bodies in the PMCo. In the hippocampal formation, NTS3 mRNA expression and immunoreactivity are both very high and selective. All subfields of Ammon's horn (CA1-3) display extremely high levels of hybridization signal. Some labeled cells are also evident in the hilus of the dentate gyrus (DG, arrowheads), whereas the DG granule cell layer itself is only faintly labeled. Scale bars = 200 μ m (a, a'), 150 μ m (b, b'), 60 μ m (c, c'), 12 μ m (inset to b), 40 μ m (inset to b'). Reprinted from [Sarret P, Krzywkowski P, Segal L, Nielsen MS, Petersen CM, and Mazella J \(2003\)](#) Distribution of NTS3 receptor/sortilin mRNA and protein in the rat central nervous system. *Journal of Comparative Neurology* 461(4): 483-505, with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Ltd.

Many of the regions enriched in NTS3 mRNA and immunoreactive neurons had previously been documented as highly innervated by neurotensinergic axon terminals, thereby supporting the concept that NTS3 may act as a *bona fide* NT receptor. As observed for the NTS2 receptor, the distribution of NTS3/sortilin did, however, largely exceed that of NT terminal fields. Several regions previously described as NT-negative, such as the hippocampal CA fields, the cerebral cortex, and the cerebellum, displayed very high levels of NTS2 and NTS3 mRNA and protein, suggesting that NT may not be the exclusive endogenous ligand to act on NTS2 and NTS3 receptors. Moreover, the superimposition of NTS3/sortilin with NTS1 and NTS2 receptor subtypes suggests that it might interact with these receptors to mediate central NT effects. At the electron microscopic level, NTS3 was predominantly concentrated over intracytoplasmic membrane-bound organelles, such as the Golgi apparatus, saccules of endoplasmic reticulum, and vesicles. This subcellular distribution conforms to the purported role of this protein in the sorting and trafficking of intracellular ligands.

NT/Dopamine Interactions

It has long been recognized that centrally administered NT exerts a number of effects similar to those of neuroleptic drugs. This observation led to a considerable number of studies aimed at defining the interactions that might exist between NT and DA systems in the brain. There is at present unquestionable neuroanatomical, electrophysiological, neurochemical, and behavioral evidence that NT modulates DA transmission in the nigrostriatal and mesocorticolimbic pathways. Actually, NT behaves either as a psychostimulant or as a neuroleptic, depending on what part of the mesocorticolimbic DA circuitry is targeted by the peptide. *In vitro* and *in vivo* application of NT to DA

neurons of the VTA stimulates DA neuronal activity. In contrast, administration of NT in the nucleus accumbens, a projection area of the VTA, inhibits DA transmission. The stimulatory, psychostimulant-like effects are mediated through NTS1 receptors that are abundantly expressed by VTA DA neuronal cell bodies and dendrites. The inhibitory, neuroleptic-like effects are also mediated via NTS1 receptors located post-synaptically to DA nerve terminals in the VTA. In addition, both antipsychotic and psychoactive drugs modify NT expression and transmission in these circuits, suggesting that the peptide mediates the effects of both types of drugs. Recent studies with either the NTS1 antagonist SR 48692 or with NT and NTS1 knockout mice further support the notion that NT participates, via the NTS1, in the response to psychostimulants as well as to that of both typical and atypical neuroleptic drugs.

Since clinical and post-mortem data indicate that central NT systems are altered in schizophrenic patients, there is an ongoing debate to know whether an NTS1 agonist or antagonist would be accurate as an antipsychotic drug in the treatment of schizophrenia. Recent evidence points to NT agonists as offering promising leads for developing an active neuroleptic-like drug. Thus, SR48692 failed in clinical trials to reveal antipsychotic properties whereas NT agonist mimetics, crossing the blood-brain barrier, proved to exert neuroleptic-like effects in the rat following peripheral administration. On the other hand, there are reasons to believe that an NT antagonist might find useful application in treating drug addicts since amphetamine and cocaine sensitization are attenuated by SR48692 and SR142948A in the rat.

NT and Pain Modulation

Considerable breakthroughs have been made in the past 5-10 years in understanding the role of NT in pain responses. Originally, intrathecal and intracerebroventricular administrations of NT were shown to produce a profound analgesia in a variety of pain paradigms, including hotplate, tail-flick, and writhing tests. It is important to note that NT's antinociceptive effects were also reported to be naloxone insensitive, implying that they were not exerted through endogenous opioid systems. Importantly, it has also been recently demonstrated that NT agonists combined with opioids may act synergistically to reduce nociception. However, site-specific microinjection experiments have provided evidence that NT may actually induce hyperalgesia instead of analgesia following administration in the periaqueductal gray and rostroventral medulla (RVM) and brain regions projecting to these structures. Indeed, studies performed using lower, close-to-physiological doses suggest that NT produces facilitatory effects on nociception. The biphasic effect of NT may be explained by a dose-dependent activation of distinct populations of pain modulatory neurons or by the recruitment of different receptor subtypes. Nevertheless, the recent demonstration that NT knockout mice display defects in stress-induced analgesia may also suggest that the transition

from pain facilitation to analgesia involves stress-induced increases in NT signaling in pain modulatory regions.

Both NTS1 and NTS2 appear to be required for different aspects of NT-induced analgesia. Knockdown of the NTS1 receptor, using either knockout mice or peptidic nucleic acids, as an antisense strategy, revealed that NT fails to increase response latencies in the hotplate test. Furthermore, intrathecal or intra-RVM administration of the relatively selective NTS1 antagonist SR48692 also partly abolished NT-induced analgesia in the tail-flick assay. Although a number of studies have implicated NTS1 in NT modulation of pain responses, several lines of evidence point to the levocabastine-sensitive low-affinity NTS2 subtype as a major antinociceptor. Indeed, NTS2-preferring NT analogs were considerably more potent in eliciting antinociception than were NTS1-preferring analogs. Among them, intrathecal or intracerebroventricular injection of the NTS2-selective agonist, JMV-431, induced strong antinociceptive responses in acute, visceral, and formalin pain tests in rodents. Similarly, intrathecal or intra-RVM administration of both selective-NTS2 analogs, levocabastine and β -lactotensin induced analgesia in rats, as measured in the tail-flick test. Accordingly, sustained delivery of antisense oligodeoxynucleotides directed against NTS2 to the brain, or spinal administration of selective NTS2 dicer-substrate small interfering RNA (DsiRNA), markedly reduced NT-induced antinociception. Accordingly, the generation of NTS2-deficient mice confirmed the involvement of this receptor in pain processing. Finally, it was recently demonstrated that both NTS1 and NTS2 agonists significantly reversed the nociceptive behaviors induced by a sciatic nerve constriction, providing evidence for a direct involvement of the neurotensinergic system in painful neuropathies. The future development of tissue-specific knockdown of both NTS1 and NTS2 will help determine their respective role in NT pain modulation. Further research to design NTS1- and NTS2-selective novel analogs that cross the blood-brain barrier may also offer new avenues for the treatment of pain without the side effects associated with the use of opioids.

Feeding Behavior and Obesity

It is well documented that food intake and body weight are regulated by a number of hypothalamic orexigenic and anorexigenic neuropeptides. These peptides are thought to coordinately regulate body weight through a complex, partially elucidated mechanism orchestrated in part by leptin, an adipose tissue-released hormone that acts centrally in the hypothalamus to control body weight and energy expenditure. The anorexigenic effect of centrally administered NT has been known for 20 years. Further support for a role of NT in food intake came from the observation that hypothalamic expression levels of NT are decreased in genetic models of obese rats and mice including the Zucker *fa/fa* rat and the *ob/ob* mouse that are deficient in leptin receptor and leptin,

respectively. More recently, a number of reports pointed to NT as a possible mediator of leptin-induced reduction in food intake. It has been shown that leptin receptors are present on and activate NT-expressing neurons in the arcuate nucleus and the dorsomedial area of the rat hypothalamus. Furthermore, central administration of leptin for 3 days increases NT gene expression whereas chronic intraperitoneal administration for 7 days decreases NT levels in the lateral hypothalamus. Leptin and NT reciprocally potentiate each other's inhibitory effect on food intake. Immunoneutralization of central NT with an NT antiserum or administration of the NT antagonist SR48692 completely blocks the inhibitory effect of leptin on food intake in food-deprived rats. Finally, in contrast to NT knockout mice that did not differ in their body weight from their wild-type littermates, NTS1 knockout mice exhibited increased food consumption, body weight, and white adipose tissue mass compared with wild-type littermates. Together, these data strongly suggest that NT is a major link in the complex neuropeptide network that controls body weight and that the peptide effects on feeding are exerted through the NTS1 receptor. NT agonists could therefore be useful in the treatment of obesity. In this context, it is of interest that an NT analog that crosses the blood-brain barrier was found to reduce food intake and body weight following intraperitoneal administration in normal and genetically obese Zucker fa/fa rats.

See also

[Dopamine](#); [Mammalian Neuropeptide Families](#); [Neuropeptide Release](#); [Neuropeptide Synthesis and Storage](#); [Neuropeptides: Discovery](#); [Neuropeptides: Enteric Nervous System](#); [Neuropeptides: Food Intake](#); [Neuropeptides: Pain](#)

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