Emerging Evidence for Neurotensin Receptor 1 Antagonists as Novel Pharmaceutics in Neurodegenerative Disorders

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Abstract: The role that the tridecapeptide neurotensin (NT) plays in the modulation of the aminoacidergic transmission is analyzed in different rat brain regions. NT exerts its effects through the activation of different receptor subtypes, NTR1, NTR2 and NTR3. The contribution of NTR1 receptor in modulating and reinforcing glutamate signalling will be shown including the involvement of interactions between NT and N-methyl-D-aspartate (NMDA) receptors. Extracellular accumulation of glutamate and the excessive activation of glutamate receptors, in particular NMDA receptors, is known to represent an important factor in the induction of glutamate-mediated neuronal damage occurring in Parkinson's disease and in pathologic events such as hypoxia and ischemia. An enhancing action of NT on glutamate-induced neurodegenerative effects is shown and NTR1 receptor antagonists could therefore become novel pharmaceutics in the treatment of neurodegenerative disease.

Key Words: Glutamate release, Glutamate-induced excitotoxicity, NMDA receptors, NTR1, Neurotensin receptor agonists and antagonists.

1. INTRODUCTION

Neurotensin (NT) [1] is a brain-gut biologically active tridecapeptide which acts as a neurotransmitter or neuromodulator of classical neurotransmitters in the mammalian central nervous system (CNS). This peptide was discovered by Dr. Leeman who noticed the presence of another vasoactive substance during the purification and the following bioassay performed to isolate and identify the peptide substance P. Thereafter, a peptide, composed of thirteen amino acids (Fig. **1**), was isolated and identified for the first time in extracts of bovine hypothalami by Carraway and Leeman [2]. It was named "neurotensin" in view of its hypotensive activity [1]. In addition to a wide distribution throughout the CNS, high concentrations of NT were also found in the gastrointestinal tract of mammals, including man. Its important function in the CNS is supported by: *i)* the differential regional distribution of NT in the brain, *ii)* the presence of NT receptors in different brain areas such as cortical, striatal and limbic regions, *iii)* sodium and calcium dependent NT release and *iv)* the involvement of NT in several electrophysiological and behavioural responses [3-6]. NT exerts its effects by interacting with three different receptor subtypes, named NTR1, NTR2 and NTR3 (*see* Section 4). The effects of NT include the well-documented interaction of the peptide with dopaminergic systems [7-10], mainly due to an antagonistic action of the activated NTR1 on dopamine D2 receptor recognition and signaling *via* an intramembrane NTR1-D2 receptor-receptor interaction [11-17].

There is growing evidence that NT may play an important role in the modulation of aminoacidergic transmission in the basal ganglia and cerebral cortex [18-23]. In particular, most *in vivo* and *in vitro* studies found that the parent peptide NT(1-13) and its biologically active carboxy-terminal fragment NT(8-13), enhance striatal, nigral and cortical glutamatergic transmission *via* the activation of local NTR1 [16,18, 23,24]. However, recently Yin *et al*. [25] observed that NT inhibits glutamatergic transmission in the dorsolateral striatum, probably by reducing presynaptic glutamate release. The authors showed that NT-induced inhibition of EPSCs involves retrograde endocannabinoid signaling, since the effect was prevented by blockade of CB1 receptors, located on the presynaptic terminals.

Evidence has accumulated that glutamate, the major excitatory neurotransmitter in the CNS of vertebrates, is an important mediator of neuronal injury. The extracellular accumulation of glutamate and the excessive activation of glutamate receptors, particularly *N*-methyl-D-aspartate (NMDA) receptors, have been postulated to contribute to the neuronal cell death associated with chronic neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases and pathologic events such as hypoxia and ischemia. Thus, endogenous compounds able to modulate gluta-

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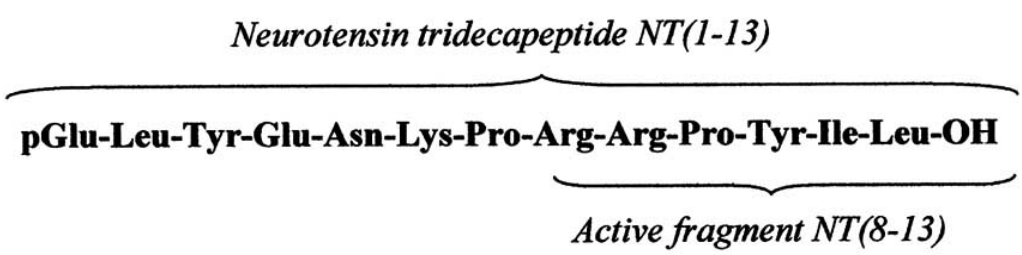


Fig. (1). Sequence of neurotensin and its active fragment NT(8-13).

mate transmission might interfere with glutamate induced cell death. In this context, reinforcing effects of NT on glutamate transmission in different brain areas and the potential modulation by NT of glutamate receptor signalling, in particular the responsiveness of the NMDA receptors, have been reported [23, 26-28]. Furthermore, NT amplifies glutamate transmission in cultured neurons from cerebral cortex [29], thus raising the hypothesis that NT might be involved in ischemic brain damage. On this background, the present mini-review will strengthen the potential relevance of NT and related compounds on glutamate-mediated excitotoxicity and neurodegenerative processes. Finally, in view of the antagonistic effect exerted by NTR1 antagonists on the NT-induced amplification of glutamate excitotoxicity, the use of selective NTR1 antagonists in combination with conventional drug treatments will be proposed as a novel therapeutic approach for the treatment of neurodegenerative pathologies.

2. STRUCTURE ACTIVITY RELATIONSHIP STUDIES ON NEUROTENSIN AND RELATED COMPOUNDS

Since the very beginning of the discovery of NT, structure activity relationship studies lead to the identification of the NT(8-13) fragment (Fig. **1**) as the minimal NT bioactive sequence [2]. Receptor binding studies of methylated NT(8–13) analogues showed that substitution at Arg9 did not significantly influence ligand affinity while exchange of Ile12 and Leu13 led to a more than 200-fold reduction of binding. On the contrary, methylation at Tyr11 produced a dramatic loss of binding affinity. The tyrosine residue in position 11 of the molecule plays a critical role in the action of NT [30-31].

A key goal in the further development of NT(8-13) analogues as potential drug candidates for treatment of schizophrenia based on the antagonistic NTR1-postjunctional D2 receptor interaction (11-17, *see* also Section 3) was the identification of a lead peptide endowed with CNS activity after peripheral administration. There is a considerable interest in the design of new drugs based on endogenous peptides which may possess potential for the treatment of pathologic conditions. Despite this potential, peptides have limited clinical applications mainly due to their poor pharmacokinetic properties. Enzymatic stability studies performed using brain membranes (for a detailed review *see* [32] demonstrated that *i*) the NT peptide bond Arg^8-Arg^9 is cleaved by endopeptidase 24.15 (EP 24.15), *ii*) EP 24.11 is entirely responsible for the Tyr^{11}-Ile^{12} cleavage, *iii*) the Pro^{10}-Tyr^{11} bond is hydrolysed by both EP 24.11 and EP 24.16. The actions of these EPs lead to complete biological inactivation of NT. In fact, the ability of NT to bind and activate its receptors is fully conserved in the C-terminal hexapeptide sequence which is the target of action of these EPs. Therefore many research efforts have been made for the development of potent NT(8-13) analogues resistant to peptidases. To this aim, amino acid replacements at the critical positions involved in enzymatic degradation were applied. It should also be underlined that such work (*see* below) on NT(8-13) analogues will be relevant for the design of novel NTR1 antagonists as novel pharmaceutics for treatment of neurodegenerative disorders, based on modification of the NT(8-13) analogue structure to give them NTR1 antagonist properties with penetration into the CNS and capability to reduce glutamate transmission and neurodegeneration (*see* Sections 7-10).

The importance of Arg^8 N-terminal modification for biological activity was explored by the researchers of Eisai Tsukuba Research Laboratories who identified the peptide [N(Me)Arg^8 tert-Leu^{12}]NT(8-13) (tert-Leu: (S)-2-amino-3,3-dimethylbutanoic acid) as a NT receptor ligand equipotent to NT(8-13) [patent Eisai, Jpn. Pat., 01-316399 (1989)]. This peptide coded as NT1 was biologically characterised *in vitro* showing agonist activity and binding affinity comparable to NT(8-13). Pharmacokinetic studies performed using [^{3}H]Pro^{10}NT1 provides supportive evidence that NT1 is able to cross the blood brain barrier after systemic administration in mice [33]. However, the most convincing indication that NT1 and other NT peptide derivatives cross the blood brain barrier is that they produce biological effects after peripheral administration that are similar to those produced following central administration.

Positions 8 and 9 were investigated substituting Arg^8-Arg^9, mainly in the NT(8-13) template, with a series of positively charged natural and non natural amino acids including ornithine and diaminobutyric acid [34]. This study suggested that chirality in position 8 as well as the length of the amino acid side chain bearing the positive charge is not crucial for NT receptor binding and activation. On the contrary, replacement of position 9 with residues with relative configuration D or the shortening of the amino acid side chain (*i.e.* from lysine to ornithine or diaminobutyric acid) is detrimental for biological activity. To prevent amino peptidase action a series of N-terminal azido NT(8-13) analogues modified at the position 8 side chain were prepared. Among them, [$hLys^8$]NT(8-13) and its N-terminal azido derivative showed binding affinity comparable to the reference peptide [35]. Recently, by combining modifications in position 8 with amino acid replacements in positions 11 and 12, the N-terminal azido peptides [N3-$hLys^8$ tert-Leu^{12}]NT(8-13) and

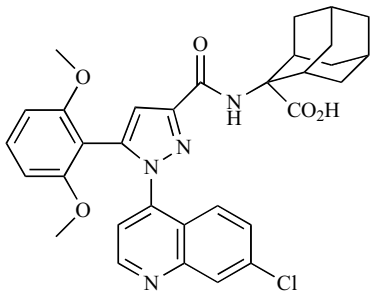
[N3-hLys^{8} Trp^{11} tert-Leu^{12}]NT(8-13) were synthesized, and found to be metabolically stable and able to elicit central biological effects after intraperitoneal administration [36]. Interestingly, the N-terminal azido moiety was demonstrated not essential for biological activity since the peptide [hLys^{8} tert-Leu^{12}]NT(8-13) behaves as NTR1 ligand with 40-fold reduced affinity compared to NT but able to induce hypothermic effects in rats after oral administration [37].

Important information for the design of novel NT analogues was acquired by modifying position 11, a position involved in the enzymatic action of both EP 24.11 and EP 24.16. D-scan investigations demonstrated that inversion of chirality in this position is not only tolerated but generated an analogue 10-fold more potent than NT [38]. Moreover the above mentioned study indicated that aromaticity in position 11 is crucial for biological activity while the oxydril moiety of Tyr^{11} is less important and can be eliminated by the replacement of Tyr with Phe [38] or with Tyr(Me) [39] without losing peptide potency. Metabolic stability investigations performed both *in vitro* and *in vivo*, demonstrated that [D-Tyr^{11}]NT and [D-Phe^{11}]NT were more resistant to enzymatic degradation than the native peptide [40] confirming the central role played by position 11 for the inactivation of NT by peptidases. Interesting NT(8-13) analogues have been obtained substituting Tyr^{11} with (2S)-2-amino-3-(1H-4-imdolyl)propanoic acid (neo-Trp), a Trp analogue in which the indole side chain has been shifted from position 3 to position 4 of the indole ring. The insertion in position 11 of neo-Trp together with modifications in position 8, 9 and 12 generated the peptide [N(Me)Arg^{8} Lys^{9} neo-Trp^{11} tert-Leu^{12}]NT(8-13), coded as NT69L, that showed high affinity for the NTR1 receptor.

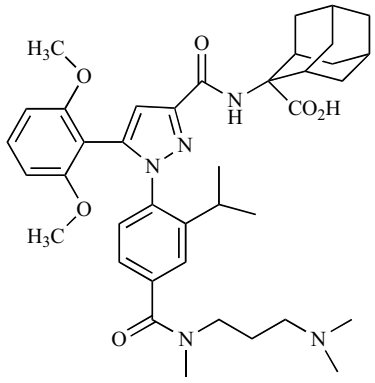
Recently [41], the cyclic peptide c[Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu] was identified as a metabolically stable NT analogue and showing nociceptive activity after oral administration in mice. Nevertheless, the authors did not provide experimental evidence on the ability of such a cyclic derivative to permeate the blood brain barrier and, thus it remains possible that the apparent central effects of these derivatives are due to indirect effects of peripheral actions.

Some of the chemical modifications discussed above, were combined for generating potent and metabolically resistant NT receptor agonists able to cross the blood brain barrier (for a detailed description of their CNS effects *see* [42]).

The first synthetic non-peptide NT receptor ligands have been reported in patent literature and described in EP0477049. They are characterized by a pyrazole scaffold substituted in position 3 with a carboxylic acid and, in general, in position 1 and 5 with aromatic nuclei. The main feature of the products described in EP0477049 is the presence at position 1 of the pyrazole ring of a phenyl, naphthyl or 4-quinolyl group, substituted or unsubstituted. These compounds, are able to displace iodinated NT from its receptor, at doses of less than one μmole, on guinea pig brain membranes. Further studies, led the Sanofi researchers to develope the compound 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)-pyrazol-3-yl)carbonylamino]tricycle-(3.3.1.1.3.7)decan-2-carboxylic acid, coded as SR 48692 (Fig. **2**), endowed with potent and selective NT receptor antagonist activity [43]. This compound competitively inhibits ^{125}I-labeled NT binding in brain tissues, taken from various species, with IC_{50} values in the nM range. Moreover, SR 48692 was demonstrated to be active *in vivo* in mice, where it was able to revert, by i.p. or oral administration, the effects induced by i.c.v injection of NT [43]. Further studies demonstrated that SR 48692 blocks many of the effects attributed to the interaction of NT with mesencephalic dopaminergic neurons. However, it failed to antagonize NT-induced hypothermia and analgesia in mouse and rat, suggesting that NT can act through a different receptor subtype, insensitive to the action of SR 48692 [44]. A detailed pharmacological characterization of SR 48692 confirms its ability to bind with high affinity both NTR1 and NTR2 [45]. In the same paper [45], the pharmacological characterization of a novel NT receptor antagonist, SR 142948A (Fig. **2**), was described in comparison with that of SR 48692. SR 142948A exhibits higher affinity than SR 48692 for both NTR1 and NTR2 but less selectivity for NTR1 over NTR2 than SR 48692. NT receptors of rat and mouse differently recognize the Sanofi compounds with high affinities for SR 142948A (1-4 nM) [45] as compared to Kd values of 82 and 300 nM for SR 48692 for rat [46] and mouse [47] NTR2 respectively. Thus, SR 142948A binds



SR48692



SR142948A

Fig. (2). Chemical formulae of standard NT receptor antagonists.

with similar affinities to the rat and mouse NTR1 and NTR2 whereas SR 48692 discriminates between both receptor subtypes and preferentially recognize NTR1. Moreover, experiments performed with human NTR2 demonstrated that SR 48692 and SR 142948A are potent human NTR2 antagonists showing Kd values of 67 and 49 nM, respectively [48]. At present, SR 142948A is considered a reference non selective NTR1 and NTR2 ligand while SR 48692 a selective NTR1 binding molecule [49].

3. BIOLOGICAL AND PHARMACOLOGICAL RELEVANCE OF NEUROTENSIN IN THE CNS

Within the CNS, NT is synthesized, stored at specific synapses and asynaptic varicosities, and, under appropriate conditions, it may be released or co-released with classical neurotransmitters. When injected in the CNS, NT elicits a wide range of biological actions including regulation of locomotion, nociception, regulation of body temperature, feeding and stimulation of anterior pituitary hormone secretion [5, 50-55]. Furthermore, NT has been shown to regulate nigrostriatal and mesocorticolimbic dopaminergic pathways [6, 56-59] as well as the activity of other neuronal systems [28, 60]. The strong interactions between NT and dopamine systems at the receptor level have raised the hypothesis that NT receptors might be novel targets for the treatment of psychoses and drug addiction [6, 58]. Peripherally, NT acts as a paracrine and endocrine modulator of both the digestive and cardiovascular systems [55]. Additionally, there is evidence to support a role for NT in growth of normal and cancerous cells [61,62]. The over-expression of NT receptors in various tumours suggested that NT-related ligands could represent valuable tools for tumour targeting [63-65].

4. NEUROTENSIN RECEPTOR SUBTYPES

Based on binding and pharmacological data, more recently confirmed by cloning experiments, it has been shown that the biological actions displayed by NT are mediated by the activation of three different specific membrane receptors, named NTR1, NTR2 and NTR3 subtypes [66-67]. All three receptor subtypes bind NT through its C-terminal hexapeptide sequence -Arg-Arg-Pro-Tyr-Ile-Leu-OH [4]. NTR1 and NTR2 belong to the family of G protein-coupled receptors (GPCRs) with seven transmembrane (TM) spanning domains [4,65,68]. Chalon *et al*. [68] reported that NTR1 and NTR2 in the rat hypothalamus show a similar length of amino acids and structural homology (43% identity and 64% homology). Dissimilarities present in their third cytoplasmic loop and in the C-terminal domain seem to be crucial to understand the molecular mechanisms underlying the different G protein coupling of NTR1 and NTR2. NTR1 and NTR2 differ also in their pharmacological properties, their affinity for NT and finally in their sensitivity to levocabastine, an antihistaminic compound. In fact, NTR1 is levocabastine-insensitive with high affinity for NT (0.1–0.3 nM) whereas NTR2 is levocabastine-sensitive with lower affinity for NT (1–5 nM) [4, 48, 69].

NTR3 is a non-GPCR and belongs to an entirely different family of proteins and in fact it is a single TM domain sorting receptor which shares 100% homology to gp95/sortilin [67].

5. INTRACELLULAR SIGNALLING PATHWAYS MODULATED BY NTR1

In the CNS and in the periphery, most of the effects induced by NT appear to be mediated by the activation of NTR1, the most extensively studied NT receptor subtype. From a molecular point of view it has been shown that in the NTR1 the binding site for the active fragment NT(8–13) lies mostly in the third extracellular loop (E3) [70]. The residues in the loop E3 involved in the interactions with NT(8–13) are by decreasing order of binding energy: Tyr347 and Phe344. E3 is connected, through TM6 and TM7, with the intracellular domain (loop I3) and the N-terminal portion of the C-terminal cytoplasmic domain that interact with the G proteins involved in signalling [71].

It has been shown that NTR1 stimulation could lead to the activation of multiple signal transduction pathways, which involves several G proteins, the expression of this potential being dependent on the host cell. In a great variety of tissues, rat brain slices [72], cultured neurons and N1E115, HT29, and NG108-15 cell line cultures [73-75] the major signal transduction pathway associated with the activation of NTR1 is the stimulation of the phospholipase C (PLC), which is responsible for the production of inositol 1,4,5-trisphosphate and Ca^{2+} mobilization [73-74, 76-77]. Molecular studies have also demonstrated that the presence of the Asp residue in the second TM of NTR1 is critical for an efficient coupling of the NTR1 to PLC. In fact, the replacement of Asp113 by an Ala residue in NTR1 strongly decreases its ability to activate inositol turnover, indicating that the functionally active conformation of NTR1 is maintained by interaction with Asp113 [78]. It has been shown that, upon activation, the receptor modulates intracellular levels of cGMP and cAMP. However, the results present in the literature are multifaceted and such a complexity might be also due to the diversity of the cells used. For example, in the murine neuroblastoma clone N1E-115, which represents a model widely used to study NT receptors, it has been shown that NTR1 activation stimulates cGMP synthesis but has no effect on cAMP metabolism [79]. However, in further studies it has been shown that the accumulation of cGMP is a consequence of the increase in intracellular calcium level, which activates a biochemical cascade, resulting in the intracellular production of nitric oxide [80]. Regarding cAMP formation both a positive [81] and negative [82] modulation of adenylyl cyclase activity by NT has been reported in certain cultured cells, whereas in rat brain slices no modulation of cAMP levels by NT has been observed [79].

6. DISTRIBUTION OF NTR1 IN THE CNS

By using different techniques such as receptor autoradiography, NTR1 mRNA *in situ* hybridization and electron microscopic analysis performed with a polyclonal antibody recognising the amino-terminal of the NTR1, it has been shown that NTR1 is widely distributed in the rat CNS [83-85]. In short, these studies indicate a relative high density of NTR1 within the substantia nigra, the ventral tegmental area, globus pallidus, the zona incerta, the suprachiasmatic nucleus, as well as in the entorhinal and retrosplenial cortices. Lower levels were found within the nucleus accumbens, septum and striatum (Table **1**) [86-87]. The presence of NTR1

Table 1. Schematic and Simplified Representation of NTR1 and NTR2 Distribution in some Nuclei of the Basal Ganglia and Mesocortical Rat Brain Regions

Brain Area	NTR1 Distribution	NTR2 Distribution
Substantia nigra	High/Middle	Low/Very low
Globus pallidus	High	Very low
Caudate/Putamen	Middle/Low	Low
Nucleus accumbens	Middle	Low
Ventral tegmental area	High	Low
Hippocampus (CA1)	Low	High
Frontal Cortex	Low	Middle/Low

Summarized from [10, 93, 94].

on axonal boutons, dendrites and spines in the basal forebrain suggests that NT may operate as a neuromodulator both at the nerve terminal and at the somatodendritic level. In addition, NT is closely co-distributed with dopamine and functional data suggest that NT modulates dopaminergic signalling in limbic and striatal brain regions mainly by antagonising dopamine D2 receptor function through a NTR1/D_2 receptor-receptor interaction located at the pre and post-synaptic level [13,15,88]. These observations suggest that NT/dopamine interactions play a relevant role in dopamine-associated neuropsychiatric and neurodegenerative disorders [6,58,89-90]. Besides the well documented modulation of the dopamine transmission by NT, there is also evidence indicating that NT, mainly *via* NTR1 modulates the release of glutamate and GABA in some brain regions. Concerning the location of NTR1 on glutamatergic terminals, the results are sparse and most of the data present in the literature indicate mostly from a functional point the presence of presynaptic NTR1 on glutamate terminals in different brain regions including the globus pallidus, striatum and cerebral cortex [21,23,91]. Of course, immunocytochemical studies at the subcellular level are important to confirm the existence of presynaptic NTR1 at glutamatergic terminals. In this context, Pickel *et al*. [92], by using immuno-gold electronmicroscopic techniques, have observed in the nucleus accumbens NTR-LI near to the plasma membrane of axon terminals that formed asymmetric excitatory type synapses with the head of dendritic spines. These represent cortical afferents most of which are glutamatergic.

7. FUNCTIONAL *IN VIVO* AND *IN VITRO* EVIDENCE FOR A MODULATION OF GLUTAMATE TRANSMISSION BY NTR1 ACTIVATION

Besides the well documented modulation of dopamine transmission by NT [8-9,13,95-96], more recent *in vivo* and *in vitro* findings indicated that the peptide enhances glutamate levels in discrete rat brain regions of the CNS such as the striatum, the substantia nigra, the globus pallidus and the medial prefrontal cortex [19,21,97-99]. *In vivo* microdialysis data indicate that NT(1-13) and its biologically active carboxy-terminal fragment NT(8-13) enhance striatal and nigral glutamate transmission *via* the activation of local NTR1 [98,99]. A dose-dependent increase of glutamate outflow induced by NT infusion into the medial prefrontal cortex has also been shown by Sanz *et al*. [97]. Furthermore, *in vitro* experiments demonstrate that in cortical slices, containing the cell-body, dendrites and collaterals of the cortical-striatal glutamate neurons, NT and its active fragment activate glutamate signalling [18]. Finally, in primary cultures of cortical neurons a calcium-dependent increase of glutamate outflow induced by the activation of NTR1 by NT and its active fragment was also found [27]. However, a recent study reported that NT inhibits glutamatergic transmission in the dorsolateral striatum, probably by reducing presynaptic glutamate release [25].

8. EVIDENCE FOR A NEUROTENSIN-INDUCED MODULATION OF NMDA RECEPTORS AND OTHER NEUROTENSIN MECHANISMS IN THE CONTROL OF GLUTAMATE RELEASE

In vitro and *in vivo* experiments recently demonstrated that the activation of NTR1 by NT amplifies the excitatory effects on glutamate transmission mediated by the activation of cortical and striatal NMDA receptors [23,27]. In particular, in cortical neuronal cell cultures the application of a subthreshold concentration of NT(1-13) enhanced the NMDA-induced increase of endogenous extracellular glutamate levels, and this effect was absent in the presence of SR 48692, a NTR1 antagonist. Furthermore, *in vivo* microdialysis studies clearly demonstrated that in the mature animal brain, which contains in respect to cell culture preparations complex integrations between several and different neuronal systems as well as interactions among neurons and glial cells, NT at a concentration by itself ineffective enhances the NMDA-induced increase of cortical and striatal glutamate extracellular levels. In both brain regions, the NT-induced amplification of the NMDA-receptor signalling is mediated by the activation of NTR1, since it is blocked by SR 48692. In line with these findings, Matsuyama *et al*. [24] showed that the NT-mediated decrease in Thr75 of DARPP-32 could be blocked by combined treatment with NMDA and AMPA receptor antagonists. This observation can be interpreted in multiple ways, including as reported by the authors that NT acts on presynaptic glutamatergic terminals and results in the

stimulation of glutamate release from corticostriatal and thalamostriatal terminals. In addition, these data could suggest that tonic glutamate transmission is required for the effect of NT as well as that NT could act to potentiate glutamate receptor function. From a mechanistic point of view, it is worth noting that the protein kinase C inhibitor, calphostin-C, prevented the effect of NT on NMDA receptor function, suggesting that the NT-mediated potentiation of NMDA receptor signalling may be mediated by phosphorylation(s) of the NMDA receptors, probably at the level of the receptor-associated protein(s) involved in receptor signalling and/or trafficking [23].

It should be noticed however that excitatory effects of NT on glutamate neurons, with increased glutamate release, may also be produced *via* a NTR1-mediated modulation of a slow non selective cation conductance involving a reduction of a membrane potassium conductance [100-101] leading to excitation also of neurons like the nigral and ventral tegmental dopamine nerve cells [100,102]. Thus, the antagonistic NTR1/D2 autoreceptor interaction and the facilitatory NTR1/NMDA receptor interaction are not the only mechanisms to produce excitation of the midbrain dopamine nerve cells. Recently, a role of calcium influx in the NT-induced excitation of the midbrain dopamine neurons has also been demonstrated [103]. However, such an increase in calcium influx could involve a NT receptor induced increase in NMDA receptor signaling. Finally, NT has also been shown to increase activity in the prefrontal GABAergic neurons [104]. Thus, in view of the excitatory effects of NT on several types of neurons, it should also be considered that enhancement of glutamate release by NT may also involve indirect excitatory effects *via* other neurons at the network or the local circuit level.

9. RELEVANCE OF NEUROTENSIN IN NEURODEGENERATIVE PROCESSES

The demonstration of a NTR1-mediated enhancement of glutamate transmission together with the *in vitro* and *in vivo* evidence for an interaction between NT and NMDA receptors, leading to an amplification of NMDA receptor signaling (Fig. **3**), raised the hypothesis that NT may play a relevant role in reinforcing the effects exerted by glutamate on a variety of CNS functions. In particular, it is well known that glutamate is an important mediator of neuronal injury and the excessive stimulation of glutamate receptors, especially NMDA receptors, can lead to excitotoxicity, which is implicated in the neuronal cell death occurring during degenerative processes. Thus, it may be postulated that NT, by enhancing glutamate signals in several brain regions, might be involved in the etiology or progression of these pathologies.

In this paragraph, the effects of NT in modulating the glutamate-induced neurodegenerative effects in cultured rat mesencephalic dopaminergic [26] and cortical [27] neurons will be briefly summarized. Mesencephalic cell cultures, which contain dopaminergic neurons, express glutamate receptor subtypes [105] as well as functional NT receptors [106], providing a suitable model for testing the influence of NT on glutamate-induced neurotoxic effects. In this *in vitro* preparation by using biochemical and morphological approaches [26], we provided evidence that the neurotoxic effects of glutamate on dopaminergic cells were exacerbated by NT. In particular, in the cultures intoxicated with glutamate, a reduction of [^{3}H]-dopamine uptake, used as an index of metabolic and structural integrity of the dopaminergic neurons in culture, was observed. This effect was exacerbated by NT, when it was applied in combination with glutamate, at concentrations by themselves ineffective. Similar results were also obtained by evaluating the vulnerability of the mesencephalic cells to glutamate using tyrosine hydroxylase (TH) immunoreactivity. The TH-immunoreactive cell counts allowed us to quantify dopamine cell survival or loss of phenotype and thus to validate the results obtained with the biochemical approach. Interestingly, the selective non-peptide NT receptor antagonist SR 48692 counteracted the effects of NT in amplifying glutamate-induced reduction of [^{3}H]-dopamine uptake and TH-immunoreactive cell number, thus indicating that NTR1 is mainly involved in the NT-induced enhancement of glutamate injury. Based on the above findings, it could be speculated that under pathological conditions NT, both by enhancing glutamate release and by amplifying the NMDA receptor signalling, may contribute to the degeneration of nigrostriatal dopamine neurons which characterizes Parkinson's disease. In this context, it is worth noting that increased NT levels were found in the basal ganglia of Parkinson's disease patients [107,108].

However, in order to have a balanced review, a complementary mechanism may be considered, such as NT antagonism of D2 receptor autoinhibition *via* a NTR1/D2 autoreceptor interaction in the dopamine nerve cells. There exists experimental support for such a mechanism. It has been shown in primary cell cultures prepared from mouse mesencephala that glutamate treatment doubled nitric oxide (NO) and superoxide radical formation, leading to dopaminergic cell degeneration and extensively altered neuronal appearance. Pretreatment with the dopamine receptor D2/D3 agonist lisuride was found to significantly reduce oxidative stress and increase the survival of dopaminergic neurons compared to glutamate-treated cultures. Such a beneficial

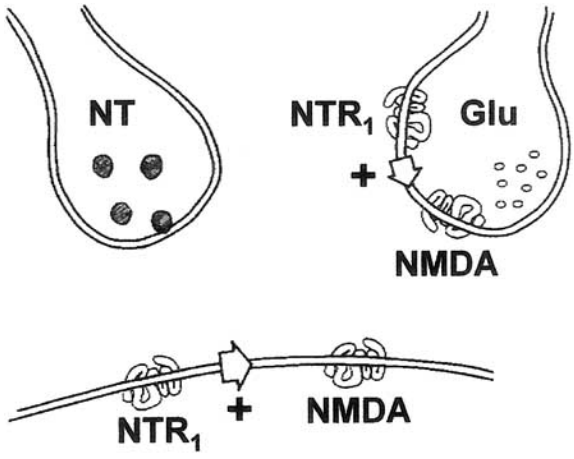


Fig. (3). Schematic representation of the possible pre- and post-synaptic intramembrane interactions between neurotensin receptor (NTR_1) and NMDA receptor, leading to an amplification of NMDA receptor signaling (for details *see* text). These scheme represents a working hypothesis on the possible molecular mechanism(s) underlying the neurotensin-induced enhancement of NMDA receptor responsiveness.

effect of lisuride was completely lost by the D2/D3 receptor antagonist sulpiride when co-treated in cultures [109]. Thus, in view of these findings it may be suggested that NT antagonism of D2 signaling could be comparable to that of sulpiride and that D2 autoreceptor activity may be essential for dopamine nerve cell survival.

A reinforcing effect of NT on glutamate-mediated excitotoxic processes has been also demonstrated in cultured rat cortical neurons. In fact, in this preparation the treatment with NT amplifies the glutamate-induced reduction of [^{3}H]-GABA uptake and increase in the number of apoptotic nuclei *via* the activation of NTR1 [27]. These findings further strengthen the evidence for a significant pathophysiological involvement of NTR1 in glutamate-induced neurodegenerative processes.

Recently, by measuring different parameters like extracellular glutamate levels, lactate dehydrogenase levels (LDH), mitochondrial dehydrogenase activity, apoptotic nerve cell death and microtubule associate protein 2 (MAP2) immunoreactivity, we provided strong biochemical and morphological evidence that NT is involved in neurodegenerative events induced by oxygen-glucose deprivation (OGD), an *in vitro* model of cerebral ischemia [29]. In fact, NT enhanced the OGD-induced increase of LDH, endogenous extracellular glutamate levels, and apoptotic nerve cell death. In addition, the peptide enhanced the OGD-induced loss of mitochondrial functionality and increase of MAP2 aggregations. These effects were blocked by the NTR1 antagonist SR 48692. In addition, the NTR1 antagonist by itself counteracted the neuronal death induced by OGD alone indicating that the blockade of NTR1 can significantly increase cell membrane integrity and cell viability. Taken together these biochemical and morphological results, suggest that cortical NTR1 activation may contribute to neuronal injury during ischemia and that NTR1 antagonists may be protective. Concerning the mechanism(s) that could underlie this effect, it could be speculated that during OGD there is an enhancement of the release of NT from neuronal and/or glial cells with a consequent increased activation of NTR1. These events could lead to the enhancement of glutamate outflow as well as of glutamate-induced neurotoxicity (*see* above). In this context, it is worth noting that in the rat the occlusion of the middle cerebral artery, which represents a model of stroke, significantly increases NT immunoreactivity in certain brain regions [110]. Such a hypothesis may provide a rationale support for the use of NTR1 antagonist(s) as potential drugs for the treatment of some disorders in which a pathological increase in NT levels could enhance glutamate release and amplify glutamate-induced neuronal damage.

10. CONCLUDING REMARKS: NEUROTENSIN/GLUTAMATE INTERACTION IN NEURODEGENERATION

In conclusion, we have reported the existence of a close interaction, both at pre and postsynaptic level, between NT and glutamate neuronal signalling. In particular, NT increases glutamate release and simultaneously amplifies the responsiveness of the NMDA receptors. The activation of NMDA receptors might, in turn, mediate a further enhancement of endogenous NT outflow. These mechanisms may represent the neurochemical substrate underlying the hypothesis for a pathophysiological role of NT in glutamate-induced neurodegeneration. In view of the antagonistic effect exerted by NTR1 antagonists on the NT-induced amplification of glutamate excitotoxicity, the use of selective NTR1 antagonists in combination with conventional drug treatments could represent a novel therapeutic approach for the treatment of neurodegenerative pathologies.

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