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**NEUROTENSIN AS MODULATOR OF GLUTAMATERGIC SIGNALLING:
RELEVANCE IN NEURODEGENERATIVE DISEASES**

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Dottorando
Dott.ssa Mazza Roberta

Tutore
Prof. Tanganelli Sergio

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ORAL COMMUNICATIONS:

I. Poster presented at the X National Congress for PhD students in Pharmacology and related sciences:
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"Neurotensin receptor involvement in the rise of extracellular glutamate levels and apoptotic neuronal cell death in primary cortical cultures after oxygen and glucose deprivation"

II. Oral communication, University of Ferrara:
March 23rd, 2007 Dep. Clinical and Experimental Medicine, Pharmacology Section, FERRARA (ITALY)
"Neurodegenerative aspects of hypoxic-ischemic brain damage: focus on the involvement of neurotensin receptor 1"

III. Oral communication at the XII National Congress for PhD students in Pharmacology and related sciences:
September 22nd-25th, 2008 Certosa di Pontignano, SIENA (ITALY)
"Neurotensin system modulation of glutamatergic signalling: relevance for the treatment of neurodegenerative diseases"

LIST OF ABBREVIATIONS

6-OHDA	**6-hydroxydopamine**
AD	**Alzheimer's Disease**
AIF	**Apoptosis Inducing Factor**
ALS	**Amyotrophic Lateral Sclerosis**
AMPA	**α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate**
BBB	**Blood-Brain Barrier**
CB1	**Cannabinoid receptor type 1**
CNS	**Central Nervous System**
DA	**Dopamine**
EAA	**Excitatory Amino Acid**
GPe	**Globus Pallidus, external segment**
GPi	**Globus Pallidus, internal segment**
HD	**Huntington's Disease**
IP3	**Phosphatidil inositole 1,4,5 phosphate**
L-DOPA	**L-dopamine**
mGluRs	**metabotropic Glutamate Receptors**
MK-801	**NMDA receptor antagonist**
NAc	**Nucleus Accumbens**
NGF	**Nerve Growth Factor**
NMDA	**2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)**
NT	**Neurotensin**
NTS1	**Neurotensin receptor type 1**
PCD	**Programmed Cell Death**
PD	**Parkinson's Disease**
PKC	**Protein Kinase C**

PLC	**Phospholipase C**
RM	**Receptor Mosaic**
ROS	**Reactive Oxygen Species**
SNc	**Substantia Nigra pars compacta**
SNr	**Substantia Nigra pars reticulata**
SR48692	**NTS1 antagonist**
STn	**Sub Thalamic nucleus**
TNF	**Tumor Necrosis Factor**
VTA	**Ventral Tegmental Area**

ABSTRACT

Rationale: Neurotensin (NT) is a tridecapeptide widely distributed in mammalian brain, where acts as a neurotransmitter or neuromodulator of classical neurotransmitters, mainly through the activation of its receptor NTS1. Several in vitro and in vivo studies have demonstrated the existence of close interactions between NT and dopamine (DA) systems both in limbic and striatal brain regions (Nemeroff CB., 1985; Binder EB., 2001; Cacéda R., 2006). Because of the involvement of an over-activation of DA system in the development of neurological disorders such as schizophrenia, psychosis and dyskinesia, a strong attention was given to the study of complex interactions between NTS1 and D2 dopamine receptor, highlighting the existence of receptor-receptor interaction, potential target for developing new anti-schizophrenic drugs (Ferraro L., 2007). In addition, neurochemical and biochemical data indicate that NT plays a crucial role in regulating glutamatergic transmission, probably inducing an amplification of NMDA receptor signalling, even at threshold concentrations (10nM) (Antonelli T., 2004).

Results I: The neuromodulatory function of NT on glutamatergic signalling was studied in an in vitro model of primary cortical cultures, highlighting a dose-dependent effect (NT 0.1-300 nM) on glutamate release. In addition, NT show the ability to amplify the NMDA-induced (100nM) increase of glutamate release. The use of the NTS1 receptor antagonists, SR48692 (100nM) and the NMDA receptor antagonist MK-801 (1μM), in combination with an effective concentration of NT, made possible to hypothesize that the mechanism involved could be an NTS1/NMDA receptor-receptor interaction (Antonelli T., 2004; Ferraro L., 2008) both at striatal and cortical level. It has been postulated that the accumulation of extracellular glutamate level and the consequent excessive activation of NMDA receptors (excitotoxic mechanism) contributes to neuronal death associated with chronic and acute neurodegenerative diseases (Olney JW., 1978). Since the data obtained to date suggest a NT-mediated strengthening on several glutamatergic functions in the central nervous system, our work was intended to deepen its possible involvement in glutamate-induced neurodegenerative mechanisms. The in vitro model of cerebral ischemia obtained by oxygen and glucose deprivation (OGD) showed a significant increase in extracellular levels of glutamate. In addition, significant alterations of biochemical and morphological

parameters measured were observed. Increase the release of LDH, reduction of mitochondrial oxidative capacity (MTT levels), increased activity of caspase-3, increased number of apoptotic (fragmented) nuclei, increasead level of AN(+)/PI(-) immunoreactive cells and MAP-2 dendritic aggregations was measured 24 hours after the ischemic insult. The addition of NT (100nM) to the culture medium showed a significant increase in the OGD-induced changes, while cells pre-exposure to the NTS1 antagonist SR48692 (100nM) blocked the effect of both the neuropeptide and OGD exposure, alone or in combination. The results obtained with this in vitro model of cerebral ischemia, stress the involvement of NT activity in the eziopathogenesis of an acute neurodegenerative disease (Antonelli T., 2008).

Results II: At basal ganglia level, NT induces an amplification of glutamate release, probably through a NTS1/D2 antagonistic interaction. This phenomenon could contribute to the degeneration of dopaminergic nigro-striatal neurons by the means of an excitotoxic mechanism, pathogenetic feature of Parkinson's disease (PD). In this contex, experiments were conducted with the in vivo microdialysis technique at striatal and cortical level, anatomical areas notoriously involved in PD. The results obtained again showed that a, potential, NTS1/NMDA receptor-receptor interaction induces a glutamatergic signalling amplification. The observed increase in glutamate extracellular levels induced by treatment with NMDA (100 and 500μM) and NT (10nM), showed once again to be partially blocked by treatment with NT antagonist SR48692 (Ferraro L., 2008). Given the potential neuroprotective role played SR48692, successive studies in an vivo model of PD achieved through unilateral lesion of the nigro-striatalpathway with the neurotoxin 6-idroxydopamine (6-OHDA) were done. Three experimental groups were tested for the turning rotation behaviour and by a challenge with NMDA 100μM: lesioned rats, rats exposed only to vehicle and lesioned rats treated with the neurotensinergic antagonist. The animals exposed to SR48692 have shown a significant recovery for both the parameter of turning behaviour and responsiveness to pharmacological challenge with NMDA (Ferraro L., 2008). The results obtained can lead to the hypothesis that the use of selective NTS1 receptor antagonists, in combination with conventional drug treatments, could provide a new terapeutic approach for chronic and acute neurodegenerative diseases treatment, such as cerebral ischemia and Parkinson's disease.

Razionale: La neurotensina (NT) è un tridecapeptide ampiamente distribuito nell'organismo dei mammiferi. A livello del sistema nervoso agisce sia come neuromodulatore che come neurotrasmettitore, interagendo con svariati sistemi di neurotrasmissione, principalmente grazie all'attività del suo recettore di tipo 1 (NTS1). Numerosi studi in vivo e in vitro hanno dimostrato l'esistenza di una specifica interazione tra il sistema neurotensinergico e quello dopaminergico sia a livello limbico che striatale (Nemeroff CB., 1985; Binder EB., 2001; Cacéda R., 2006). In considerazione del coinvolgimento di un'iperattivazione dopaminergica a livello mesolimbico nello sviluppo di alcune patologie neurologiche quali schizofrenia, psicosi e discinesie, una forte attenzione è stata rivolta allo studio delle complesse interazioni tra recettore NTS1 ed il recettore dopaminergico D2, evidenziando l'esistenza di un'interazione recettore-recettore, potenziale target per lo sviluppo di nuove terapie anti-schizofreniche (Ferraro L., 2007). In oltre, dati biochimici e neurochimici indicherebbero che la NT svolge un ruolo cruciale anche nella regolazione del segnale glutammatergico, inducendo un'amplificazione della funzionalità del recettore glutammatergico NMDA, anche a concentrazioni soglia (10nM) (Antonelli T., 2004).

Risultati I: Il ruolo neuromodulatorio della NT sull'attività glutammatergica è stato studiato in un modello di colture corticali primarie, evidenziando un effetto dose-dipendente (NT 0.1 - 300nM) sull'aumento del rilascio del glutammato stesso. In oltre, la NT è risultata essere in grado di amplificare l'aumento del rilascio di glutammato mediato dall'agonista selettivo per i recettori NMDA (NMDA 100nM). L'utilizzo degli antagonisti per il recettore NTS1, SR48692 100nM e per il recettore NMDA, MK-801 1μM, in associazione ad una concentrazione efficacie di NT, ha permesso di ipotizzare che il meccanismo alla base dell'effetto visto sia l'esistenza di un'interazione NTS1/NMDA di tipo recettore-recettore (Antonelli T., 2004; Ferraro L., 2008). E' stato postulato che l'accumulo di glutammato a livello extracellulare e l'eccesso di attivazione dei suoi recettori NMDA (eccitotossicità), contribuisca alla morte neuronale associata a malattie neurodegenerative croniche e acute (Olney JW., 1978). Poiché i dati sino ad oggi ottenuti, suggeriscono un rafforzamento NT-mediato su diverse funzioni esercitate dal glutammato nel sistema nervoso centrale, abbiamo voluto approfondire in una seconda serie di esperimenti il suo possibile coinvolgimento nei meccanismi neurodegenerativi glutammato-indotti. Sono stati quindi condotti esperimenti in vitro su colture di neuroni corticali di ratto esposte a deprivazione di ossigeno e glucosio (OGD), modello in vitro di ischemia cerebrale. Tale modello ha evidenziato un aumento significativo dei livelli extracellulari di glutammato. In oltre, alterazioni significative dei

parametri biochimiche e morfologici valutati sono state osservate: aumento del rilascio di LDH, alterazioni della capacità ossidativa di mitocondri (livelli di MTT), aumento di attività dell'enzima caspasi-3, aumento del numero dei nuclei apoptotici, aumento di cellule AN(+)/PI(-) Immunoreattive e del numero di aggregazioni MAP-2 a livello dendritico, misurata 24h dopo l'insulto ischemico. L'aggiunta di NT (100nM) ha evidenziato un significativo aumento di tutte le alterazioni OGD-indotte, mentre la pre-esposizione delle cellule all'antagonista neurotensinergico SR48692 (100nM) ha bloccato sia l'effetto del neuropeptide e che del trattamento di OGD, da soli o in combinazione tra loro. I risultati ottenuti con questo modello in vitro di ischemia cerebrale sottolineano il coinvolgimento di NT in eventi patologici acuti di tipo ischemico (Antonelli T., 2008).

Risultati II: A livello dei gangli basali, la NT è in grado di amplificare il rilascio di glutammato, verosimilmente grazie ad un'interazione di tipo antagonistico NTS1/D2. Tale fenomeno potrebbe contribuire alla degenerazione dei neuroni dopaminergici nigro-striatali indotta da un'eccessiva attivazione del sistema glutammatergico, caratteristica eziopatogenetica del morbo di Parkinson (PD). A tale proposito sono stati condotti esperimenti di microdialisi in vivo a livello del tessuto striato, area notoriamente coinvolta nel PD, e della corteccia cerebrale. I risultati ottenuti hanno evidenziato come, anche in questo caso, vi sia una potenziale interazione NTS1/NMDA di tipo recettore-recettore che comporta un'amplificazione del segnale glutammatergico. L'aumento dei livelli extracellulari di glutammato indotto sia dal trattamento con il solo NMDA che dalla co-esposizione con NT, risultava ancora una volta essere bloccato parzialmente dal trattamento con l'antagonista neurotensinergico SR48692 (Ferraro L., 2008). Visto il potenziale ruolo neuro protettivo svolto dall'SR48692, gli studi condotti stanno proseguendo su un modello in vivo di PD ottenuto tramite lesione unilaterale della via nigro-striatale con la neurotossina 6-idrossidopamina (6-OHDA). Una parte degli animali lesionati è stata trattata con l'antagonista neurotensinergico dando così luogo a tre gruppi di animali: esposti al solo veicolo, lesionati con 6-OHDA, lesionati con 6-OHDA e trattati con SR48692. Gli animali esposti a SR48692 hanno ad oggi evidenziato un significativo recupero sia per il parametro del *turning behaviour* che nella responsività al challenge farmacologico con NMDA 100μM (Ferraro L., 2008). Alla luce dei dati ottenuti quindi è possibile ipotizzare che l'uso di antagonisti selettivi del recettore NTS1, in associazione ai trattamenti farmacologici convenzionali potrebbe fornire un nuovo approccio per il trattamento di patologie neurodegenerative croniche e acute quali l'ischemia cerebrale e il morbo di Parkinson.

INTRODUCTION

Glutamatergic system activity is essential for daily life, controlling the correct ontogenesis of the central nervous system (CNS) by regulating synaptogenesis, neuron migration and differentiation whereas, in the postnatal life, it controls crucial CNS functions like learning, memory, motor neuron activity etc.. . As a direct consequence of its physiological essential role, glutamate release from cells and cytoplasmic organelles is a highly regulated process. When glutamate physiological aequilibrium results altered, an increase in its extracellular levels can induce cell death. Glutamate can induce neurotoxic effects particularly when neurons are coincidentally subjected to adverse conditions such as reduced levels of oxygen and glucose, increased level of oxidative stress, exposure to pathogenic agents or toxins or disease-causing genetic alterations. In these cases, an over-activation of glutamate receptors can trigger the death of neurons by "excitotoxic" mechanisms: in fact, glutamate is the principal excitatory neurotransmitter in the mammalian CNS and the activation of his receptor system lead to calcium cytoplasmic influx and release from organelles, the first element of a complex cascade of postsynaptic events that can induce neurodegeneration.

Several neurotransmitter or neuromodulator systems can control physiological glutamatergic activity. Consequently, new therapeutic opportunities arising from the identification of pharmacological targets, both in acute and chronic age-related disorders, where clinical and experimental evidences suggest an involvement of the excitotoxicity mechanisms, like epilepsy, stroke, traumatic brain and spinal cord injury,

Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) disease, amyotrophic lateral sclerosis (ALS) and several psychiatric disorders like schizophrenia.

In this context, the work presented was intended to highlight a possible role for the neuropeptide Neurotensin (NT) as glutamatergic modulator. NT central activity was widely studied in consideration of his role in Dopaminergic signalling modulation, but recently evidence grown about his physiological role as glutamatergic modulator. Particularly, it seems that NT, acting trough his receptor NTS1, can amplify the NMDA receptor activity. Given the potential involvement of this receptor in neurodegenerative mechanisms, the identification of neurotensinergic mechanism of action could be of great importance in the treatment of chronic and acute degenerative disorders of CNS.

1. NEURODEGENERATION

Neurodegenerative diseases are characterised by a net loss of neurons from specific regions of CNS. Progressive cell loss in specific neuronal populations is often associated with typical protein aggregations patterns, a pathological hallmark of neurodegenerative disorders, but the nature, time course and molecular causes of cell death and their relation to cellular alterations are still unresolved.

Two major mechanisms of neuronal death have been discussed in neurodegeneration: apoptosis and necrosis. These cell death types are different, frequently divergent, but sometimes overlapping cascades of cellular breakdown. Due to modulation of these cascades by cellular available energy, cells may use diverging execution pathways of demise. The apoptosis phenomenon was discussed in several neurodegenerative disorders, in human brain as well as in animal and cell culture models. Recently, a new picture is beginning to emerge suggesting that, in addition to apoptosis, other forms of programmed cell death may participate in neurodegeneration.

Apoptosis is a specific form of gene-directed programmed cell death (PCD) that executes removal of unnecessary, aged or damaged cells and is characterized by distinct morphological and biochemical features. It is carried out by an intrinsic suicidal machinery of the cell and can be triggered by environmental stimuli leading to DNA damage like oxidative stress, toxins, viruses, withdrawal of neurotrophic support, etc.. In *post mortem* human brain of patients with neurodegenerative disorders, e.g. AD, PD, ALS, HD etc., dying neurons are present, showing morphological features of apoptosis. These include cell shrinkage, chromatin condensation, DNA

fragmentation, and an altered expression of both proapototic and antiapoptotic proteins and DNA repair enzymes.

1.1 Glutamate neurotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and is present in millimolar concentrations in central gray matter. Once released, postsynaptic responses occur via distinct metabotropic and ionotropic receptors. Metabotropic receptors mediate their actions through GTP-binding protein dependent mechanisms that cause mobilization of Ca^{2+} from internal stores. Ionotropic receptors are associated with an ion channel, and include the *N*-methyl-d-aspartate (NMDA) receptor, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) receptor, and kainate receptor subtypes. Ionotropic receptor activation leads to membrane permeability to sodium, potassium, and/or calcium ions.

Experiments by Lucas and Newhouse (Lucas DR., Newhouse JP., 1957), which showed that L-glutamate injections could destroy the inner layers of the mouse retina, were the first to suggest that glutamate could act as a neurotoxin. These observations were replicated and expanded by Olney (Olney JW., 1978), who confirmed the retinotoxicity of glutamate and further indicated that the structurally related compound kainate produces brain lesions in immature animals that do not possess a fully developed blood–brain barrier (BBB). Olney also reported that the glutamate-induced retinotoxicity is accompanied by rapid cellular swelling which is most pronounced near dendrosomal components that are currently known to express excitatory amino acid (EAA) receptors. In 1969, he coined the term *excitotoxicity*, to indicate neurodegeneration mediated by EAAs (Olney JW., 1969). Further evidence for the excitotoxic hypothesis came from the observation that there was a close correlation between amino acids that can

induce neuronal lesions and neurotransmitters able to activate excitatory amino acid receptors like Kainate and many others (Doble A., 1999).

Initially, it was considered that the excitotoxic insult was a typical example of necrotic cell death characterised by osmotic swelling and cell lysis. In the last ten years increasing evidences suggest that, apoptotic and necrotic cell death are complementary mechanisms and that dying neurons may switch from one to the other depending on the intensity of the insult: a mild glutamate insult leads to the apoptotic pathway whereas an intense glutamate insult induces predominantly the necrotic pathway (Choi DW., 1996; Cheung NS., 1998).

The model of "classical" excitotoxicity evoked by increased levels of glutamate, have implicated the NMDA receptor subtype as being the principal vehicle of excitotoxic damage (Waxman EA., 2005), characterized by a great loss of dendritic spine were NMDA receptors are mainly localized (Arundine M., 2003). NMDA receptors are hetero-oligomeric protein composed of an NR1 subunit and at least one isoform of the NR2 subunit. The NR1 subunit appears to be costituent of all NMDA receptors, essential for a correct neuronal activity and functional NMDA receptors. The NR2 subunit exists in four isoforms (NR2A-NR2D) whom expression is developmentally regulated. It is widely documented how immature cultured neurons are less vulnerable to NMDA neurotoxicity (Marks JD., 1996; Whal P., 1989). That vulnerability changes probably arise from the different expression of NR2 subunits: NR2A and NR2C subunit are the most abundant in the adult CNS whereas NR2B and NR2D are predominantly expressed during pre- and peri-natal phases (Cull-Candy S., 2001), but the exact correlation between NR2 isoform expression and excitotoxic effect must be clarified (Steigerwald F., 2000; Mizuta I., 1998). Moreover, different NR2 isoforms can lead to functionally distinct NMDA receptors, NR1/NR2B

receptors are frequently located at extrasynaptic level whereas NR1/NR2A/NR2B receptors are frequently expressed as synaptic receptors in the forebrain (Stocca G., 1998; Tovar KR., 1999).

NMDA receptors allow the entry of sodium and calcium ions and the efflux of potassium ions. At resting membrane potentials, they are blocked by magnesium ions (Mg^{2+}), blockage that can prevent the activation by glutamic acid. When neurons are depolarised by others excitatory mechanisms (e.g. AMPA/Kainate rapid excitatory transmission) the Mg^{2+} ions can be removed by a voltage-dependent mechanisms and NMDA receptors can be activated by his ligand, leading to Na^{+} cytoplasmic influx. In order to maintain ionic equilibrium the entry of sodium ions is followed by a passive entry of chloride anion (Cl^{-}) and, following the osmotic gradient, by water entry. These three events can be identified as the first step of a cascade of cellular events that eventually lead to cell death because of an osmotic cellular swelling and a diluition of cytoplasmic contents, able to induce disruption of organelles, cell lysis and release of cell contents into the extracellular space. Nevertheless, is the calcium-dependent part of the excitotoxic cascade that appears to be a key factor in determining the final fate of the cell, essential for the toxic effect.

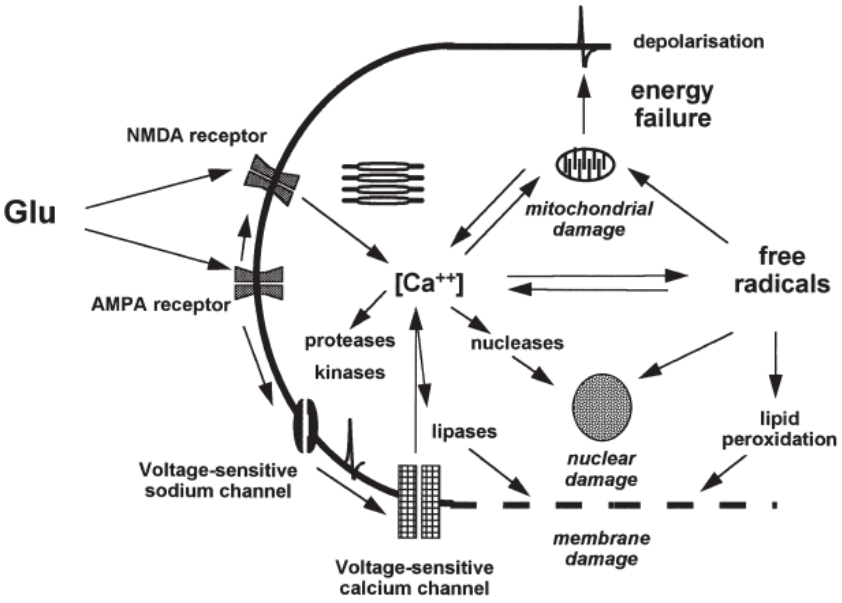


Fig. 1. Mechanisms whereby glutamate increases cytoplasmic Ca^{2+} concentrations, and modulation by endoplasmic reticulum (ER) and mitochondria. Binding of glutamate to AMPA receptors and kainate receptors opens the receptor channels resulting in Na+ influx and consequent membrane depolarization and opening of voltage-sensitive Ca^{2+} channels. Some forms of AMPA receptor are also permeable to Ca^{2+}. Binding of glutamate to NMDA receptors under depolarizing conditions opens the NMDA receptor channel resulting in large amounts of Ca^{2+} influx. Activation of metabotropic glutamate receptors (not shown) induces IP3 production and activation of IP3 receptor channels in the ER membrane resulting in release of Ca^{2+} from the ER into the cytoplasm. The increases in cytoplasmic Ca^{2+} levels in response to glutamate receptor activation can induce Ca^{2+} uptake into the mitochondria that, if excessive, can induce the production of reactive oxygen species (free radicals) and inhibit ATP production. By activating proteases and inducing oxidative stress, Ca^{2+} is a key mediator of excitotoxic cell death. (Adapted from: Doble A., 1999)

1.2 Excitotoxic cell death

1.2.1 Calcium and cell death

Ca ions (Ca^{2+}) under physiological conditions govern a multitude of cellular processes, including cell growth, differentiation, synaptic activity, plasticity, neurite outgrowth and synaptogenesis. Consequently, homeostatic mechanisms exist to maintain a low intracellular Ca^{2+} concentration so, that signal, remain spatially and temporally localized. This allows multiple independent Ca^{2+} - mediated signalling pathways to occur in the same cell. In

excitotoxicity, excessive synaptic release of glutamate can lead to the deregulation of Ca^{2+} homeostasis. Glutamate activation of postsynaptic ionotropic receptors lead to the opening of their associated ion channel, allowing the influx of Ca^{2+} and Na+ ions (Figure 1). Although physiological elevations in intracellular Ca^{2+} are essential to normal cell functioning, the excessive influx of Ca^{2+} together with any Ca^{2+} release from intracellular compartments can overwhelm Ca^{2+}-regulatory mechanisms and lead to cell death.

During aging, and particularly in neurodegenerative disorders, cellular Ca^{2+}-regulating systems are compromised resulting in synaptic dysfunction, impaired plasticity and neuronal degeneration. Oxidative stress, perturbed energy metabolism and aggregation of disease-related proteins (amyloid β-peptide, *α*-synuclein, huntingtin, etc.) adversely affect Ca^{2+} homeostasis by mechanisms that have been elucidated recently (Figure 2). Alterations of Ca^{2+}-regulating proteins in the plasma membrane (ligand- and voltage-gated Ca^{2+} channels, ion-motive ATPases, and glucose and glutamate transporters), endoplasmic reticulum (presenilin-1, ryanodine and inositol triphosphate receptors), and mitochondria (electron transport chain proteins, Bcl-2 family members, and uncoupling proteins) are implicated in age-related neuronal dysfunction and disease.

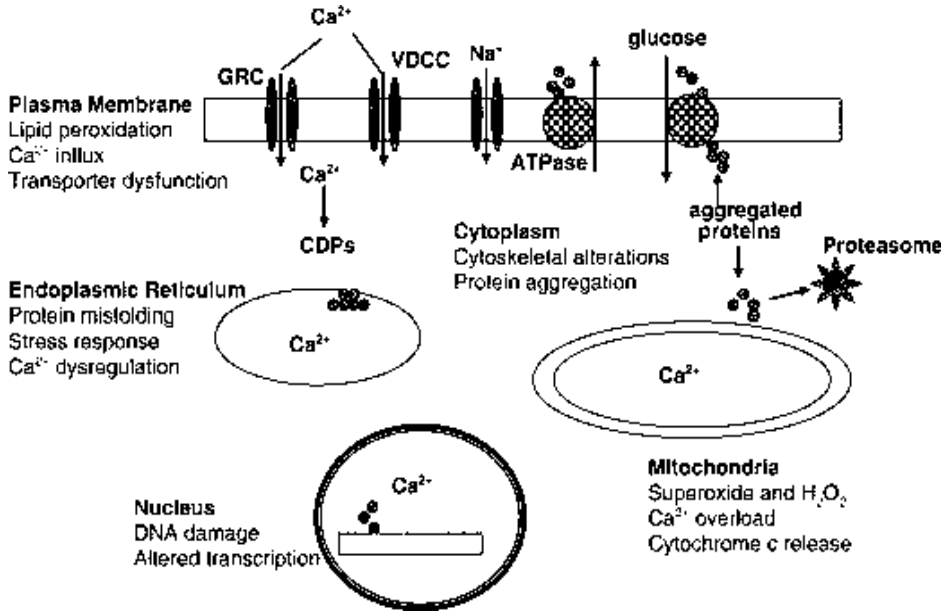


Fig. 2. Subcellular systems involved in the disruption of neuronal Ca^{2+} homeostasis in aging and neurodegenerative disorders. Oxidative stress resulting from the aging process and disease-specific mechanisms causes peroxidation of lipids in the plasma membrane that impairs the function of ion-motive ATPases and glucose transporter proteins. This promotes membrane depolarization and cellular energy depletion, which results in excessive Ca^{2+} influx through glutamate receptor channels (GRC) and voltage-dependent Ca^{2+} channels (VDCC), and accumulation of Ca^{2+} within the cell. Perturbed Ca2+ homeostasis may also result from endoplasmic reticulum stress and mitochondrial dysfunction. Abnormal aggregation of proteins such as Aβ and α-synuclein likely contribute to the damage and dysfunction of Ca^{2+}-regulating systems. Impaired function of the proteasome may contribute to the accumulation of protein aggregates throughout the cell. Excessive amounts of Ca^{2+} within the neuron can cause dysfunction of a myriad of cellular processes, including mitochondrial oxidative phosphorylation, protein production, and proper folding in the endoplasmic reticulum, and transcriptional regulation in the nucleus. Perturbed Ca^{2+} homeostasis may first adversely affect synaptic plasticity, followed by degeneration and death of neurons. Calcium dependent proteases (CDPs). (Adapted from: Matteson MP., 2007)

About the mechanisms involved in Ca^{2+}-induced cell death, considerable evidence for several different, cross-amplifying, cascades has been obtained (Figure 2). Ca^{2+} activates cysteine proteases called calpains that degrade a variety of substrates including cytoskeletal proteins, membrane receptors, and metabolic enzymes (Bi X., 1996; Caba E., 2002; Guttmann RP., 2002). Calpains may also play an important role in the triggering of apoptotic

cascades by virtue of their ability to activate caspases (Leist M., 1997; Volbracht C., 2001).

Moreover, Ca^{2+} induces oxidative stress. This occurs through several different mechanisms, including activation of oxygenases such as those in the arachidonic acid metabolism cascade (Goodman Y., 1994), perturbation of mitochondrial calcium and energy metabolism (Sengpiel B., 1998), and induction of membrane lipid peroxidation (Goodman Y., 1996). The reactive oxygen species (ROS) generated in response to glutamate-induced Ca^{2+} influx include superoxide anion radical, hydrogen peroxide, hydroxyl radical, and peroxynitrite (Culcasi M., 1994; Mattson MP., 1995; Dawson VL. & Dawson TM. 1998; Yu ZF, 1998). Finally, Ca^{2+} triggers apoptosis, a form of programmed cell death (Ankarcrona M. 1998), essentially by Ca^{2+}-mediated induction/activation of pro-apoptotic proteins such as Bax, Par-4, and p53 leading to mitochondrial membrane permeability changes, release of cytochrome c and caspase activation (Duan W. , 1999; Culmsee C., 2001; Dargusch R., 2001).

1.2.2 Oxidative stress and cell death

Several studies have reported an increase in oxidative stress that is an excessive production of ROS, in neurons subjected to excitotoxic insults performing in vivo and in vivo experiments. This phenomenon is a physiological age-related process, consequence of the normal oxidative metabolism, and several antioxidant systems like Catalase, Superoxide dismutase and Glutathione peroxidase are spent in order to eliminate them. Such molecules are superoxide radical ($O_2^{\bullet -}$), peroxynitrite (NO_3^-), hydrogen peroxyde (H_2O_2), hydroxyl radical ($OH^{\bullet -}$). If they are not efficiently eliminated can induce membrane lipids peroxidation, impare key proteins functions, damage DNA and RNA leading to cell death.

A non-physiological cause of ROS increment is a loss in Ca^{2+} homeostasis. That ion can activate several enzymes like Phospholipase A2 (PLA_2), Xantine Oxidase (XO), neuronal Nitric Oxide Syntase (nNOS, figure 3) that can produce several kinds of ROS (Gilgun-Sherki Y., 2002; Skulachev V., 2001; Rego AC., 2003)

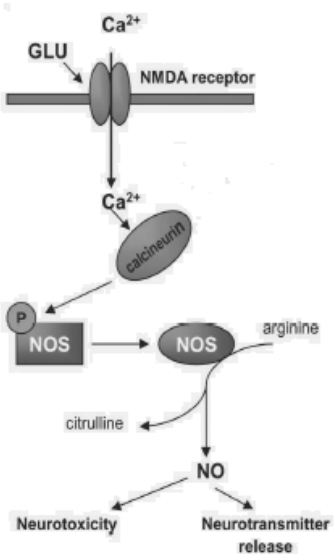


Fig. 3: Calcineurin is a Ca^{2+}-dependent protein that can induce the activation of NOS by dephosphorilation. This phenomenon is normally provided for NO physiological release mechanisms. It can also act as pathological way of neurotoxicity following an excess of Ca^{2+} at cytoplasmic level. (Adapted from: Kaminska B., 2004)

While oxidative stress may contribute to the excitotoxic process, it can also render neurons more vulnerable to excitotoxicity itself. Several studies have shown that neurons are more easily damaged by glutamate when there is an increase in oxidative stress. The membrane lipid peroxidation that follow an oxidative insult, can impair the function of key proteins involved in the maintenance of cell physiological activity and in the maintenance of cellular calcium homeostasis, generating an harmful loop. The worst and latter effect of membrane lipid peroxidation is the destabilization of Ca^{2+} cytoplasmic balance, resulting in a generalized increase in Ca^{2+} concentration following

activation of NMDA receptors, phenomenon particularly pronounced at synaptic terminals (Keller JN., 1997). Besides, Nitric oxide and other ROS may disrupt neuronal calcium homeostasis independently from lipid peroxidation (Brorson JR., 1997).

Considering that most neurodegenerative age-related disorders are generally associated with a ROS increase, it is not always clear if oxidative stress is a cause or a consequence of the excitotoxic damage. Nevertheless, it has been demonstrated in various cell culture and in vivo models that treatment with antioxidants like vitamin E, estrogens and lipoic acid protect neurons against glutamate toxicity and ischemia-like insults (Goodman Y., 1996; Wolz P., 1996).

1.2.3 Programmed cell death

In the 1905, Studnick made the first description of a developmental neuronal cell death (Studnick FK., 1905) and only 50 years later Levi-Montalcini can prove that such physiological cell death can be inhibited by a cytosolic growth factor as NGF (Levi-Montalcini R., 1966). The idea of a predetermined pathway by which specific cells die, born in 1964 when Lockshin coined the term programmed cell death (PCD) (Lockshin RA., 1964). After that, Kerr & coll. coined the term apoptosis to describe a characteristic set of cell deaths that occurs in several physiological and pathological cellular events (Kerr JF., 1972) that has been equated with PCD.

The balance between cell death and cell survival is a highly regulated process, controlled by a wide pattern of intracellular signalling. The recent discovery of neurogenesis in the adult nervous system has dramatically changed the study of eziopathogenetic mechanisms of neurodegenerative disorders and of new therapeutic strategies considering the possibility that the nervous system has an intrinsic capacity for repair. Evidences suggest

that neurogenesis is impaired in neurodegenerative disease such as PD, AD and ALS and enhanced following stroke (Jin K., 2001; Ohab JJ., 2008).

Since recently, cell death has been divided into two general groups: apoptosis, the only form of PCD known, and necrosis the passive (non programmed) form of cell death. Actually, programmed and passive cell deaths are believed to be the extreme points of a single process (Leist M., 2001).

Apoptosis

Also referred as type I PCD, apoptosis is probably the most well characterized type of PCD. Is an essential physiological process that allows critical events like embryonic development, tissue modelling, immune system development and elimination of damaged/altered cells. Besides, altered apoptotic pathways are frequently present within several pathological conditions, even in neurodegenerative diseases. Morphologically, apoptotic cells typically round up, form blebs, undergo chromatin condensation, nuclear fragmentation, breaking-off of cellular fragments called apoptotic bodies, DNA fragmentation, proteolytic cleavage of specific substrate and phosphatidylserine residues extracellular exhibition (Kerr JFR et al., 1972; Martin SJ., 1995). Those cellular events are useful to allow a rapid phagocytosis, to prevent inflammation responses consequent the release of cytosolic content in the extracellular space. The biochemical pathways employed to carry out the apoptotic event can allow a distinction between two principal apoptotic ways: intrinsic and extrinsic pathways.

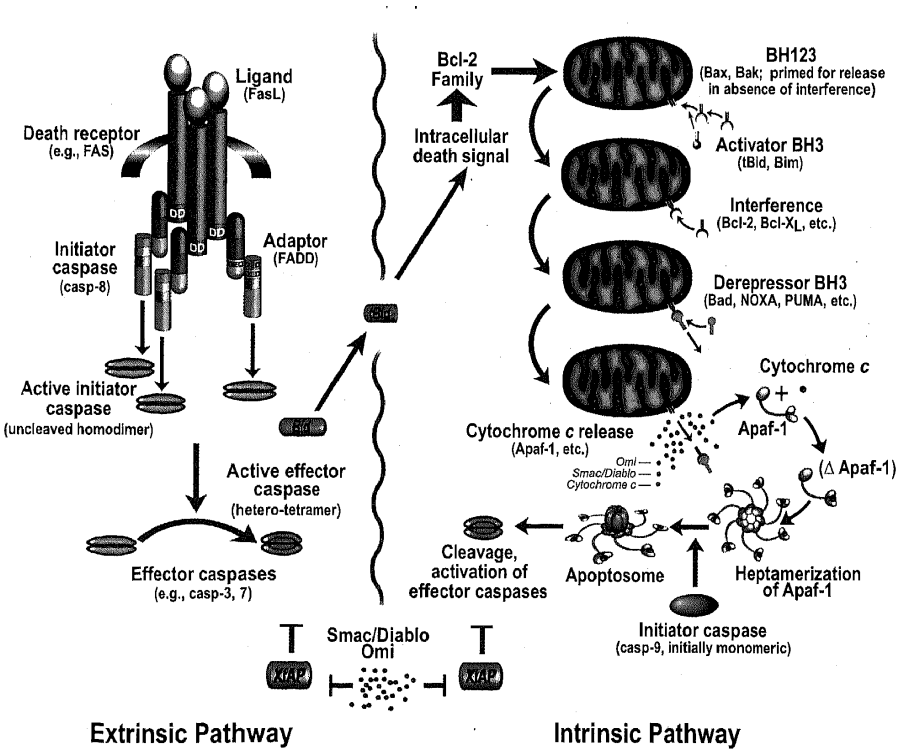


Fig. 4: Scheme of intrinsic and extrinsic pathways of apoptosis (Adapted from Bredesen DE., 2008).

The extrinsic way is characterized by the activation of specific "death receptors" like TNF receptor and Fas receptor and the involvement of a set of cell-suicide cysteine/aspartyl-protease called Caspases (Raff M., 1998; Thornberry NA., 1998), responsible for the DNA and protein fragmentation (Figure 4). The intrinsic pathway requires the involvement of specific family proteins and mitochondrial release of cytochrome C (Figure 4). The intrinsic pathway show three distinct phases:

I. *Induction Phase*: One or more death stimuli activate several members of Bcl-2 – family proteins like Bcl-2 subfamily and Bax subfamily proteins, that can induces the alteration of mitochondrial external

membrane and the release of others pro-apoptotic mitochondrial proteins (Zamzami N., 2001; Basanez G ., 1999; Pavlov EV et al. 2001)

II. *Mitochondrial Phase*: Characterized by the collapse of mitochondrial membrane potential and consequent lost of mitochondrial integrity, followed by apoptogenic factors release like: cytochrome C, AIF, endonuclease G and Smac/DIABLO (Du C., 2000; Verhagen AM., 2000)

III. *Executive Phase*: The apoptogenic factors can tie up others cytoplasmic proteins like Apaf-1, pro-caspase 9 and IAPs leading to Caspases activation (Susin SA., 1998)**.** Caspases are Cysteine Aspartate-specific protease that can be subdivided in two classes, initiator Caspase (-2 -8 -9 -10) and effector Caspase (-3 -6 -7). The first one, with cascade-mechanism, can activate the second one (Boatright KM., 2003) leading to the cleavage of specific substrate and finally to proteolysis, alteration of cell structure, inactivation of repair mechanisms, internucleosomal DNA cleavage, phagocytic uptake and many others (Thornberry NA ., 1998).

Alternative mechanisms of programmed cell death

Evidences growing about the existence of non-apoptotic forms of PCD (Foghsgaard L., 2001; Sperandio S., 2000). A large number of authors have shown that there are caspases-independent PCD mechanisms that can still present some apoptosis-like features like membrane blebbing (Foghsgaard L., 2001). One of these emergent mechanisms of death is *Autophagy*, a multifunctional process that occurs in a wide range of organisms. Autophagy includes three distinct processes: macroautophagy, microautophagy and chaperone-mediated autophagy, all characterized by a degradation pathway that complements the proteasomal pathway by degrading long-lived

proteins, protein aggregates and organelles using the lysosomal machinery (Bredesen E., 2008).

Actually, even if none of the non-apoptotic forms of PCD have gained general acceptance, PCD mechanisms can be classified on molecular, biochemical and morphological features on four principal classes (Leist M., 2001**;** Clarke PG., 1990)**:**

I. Apoptosis

II. Apoptosis-like

III. Necrosis-like

IV. Accidental necrosis

Evidence for caspase activation in neurodegenerative disease have been obtained (Friedlander RM., 1997; Galvan V., 2006) as well as some neurodegenerative models seems to demonstrate non-apoptotic form of PCD (Turmaine M., 2000). Determining which PCD pathways are triggered in each neurodegenerative dysfunctions should shed light on the degenerative process itself and its potential treatment or prevention.

1.3 Acute neurodegenerative disorders

1.3.1 Hypoxic-ischemic brain damage

Stroke is a transient or permanent reduction in cerebral blood flow, in most cases caused by the occlusion of a cerebral artery. Is a major cause of disability and death in elderly populations worldwide (Ingall T., 2004). Because of the reduction in oxygen and glucose supply, direct consequence of a stroke is a hypoxic-ischemic brain damage.

Oxygen and glucose are fundamental substrates for energy production, especially in brain tissue. Their reduction, subsequent an impairment in cerebral blood flow, impairs the energy required to maintain ionic gradients and energy-dependent processes such as presynaptic reuptake of excitatory

amino acids The consequent elevations of glutamate extracellular levels can lead to excitotoxic damage by necrotic and apoptotic mechanisms. In fact, several authors have reported that NMDA receptor antagonists can lead to neuroprotection against hypoxic brain lesions. (Simon RP., 1984), suggesting that its activation by endogenous glutamic acid released during ischemia can play a crucial role in the determination the extent of neurodegeneration. However, several clinical trials of NMDA receptor antagonists in human stroke patients failed to produce a statistically significant effect (Davis SM., 2000; Lees KR., 2000; Legos JJ., 2002), probably because of the highest individual variability between humans in respect of the more uniform ischemic animal models.

Two principal rodent models for human disease are global ischemia and focal ischemia (Lipton P., 1999):

I. *Global ischemia* involves a short (usually 15 minutes or less) very intense insult in which ATP is severely lowered, characterized by a slow development of cell death during reperfusion

II. *Focal ischemia* is a far more complex insult, there are many variations of the model including whether or not there is reperfusion, where the lesion is, the strain of rat (and the degree of temperature control in *in vivo models*)

Hypoxia/ischemia is essentially a focal insult considered a very good *in vivo* model for major forms of neonatal metabolic brain damage. Three principal types of cell death occur: a necrotic pathway, an apoptotic-like pathway and autophagy. Many experimental models are used to study ischemic damage. Primary neuronal/glial cultures from cortex (Griffin S., 2005), hippocampus (Weiss J., 1986), cerebellum, and hypothalamus (Meyers KM., 1995) of embryo or perinatal rats and mice have been used extensively to study anoxic or ischemic damage since 1983 (Rothman S., 1984). Damage usually

develops 8–24 h after 30- to 60-min in vitro ischemia and is generally monitored as a gross increase in membrane permeability to dyes or protein, in particular leak of LDH or non-exclusion of trypan blue or propidium iodide. More recently, apoptotic and necrotic changes have been monitored by standard methods of nuclear staining.

A major difference from damage in in vivo models, is the very long duration of oxygen and glucose deprivation that is generally required to induce cell death. Although 30- to 60-minutes deprivation is not long for focal insults, the degree of oxygen and glucose deprivation that cultures are exposed to is similar to that in global ischemia, where 5-10 min at 36°C produces profound delayed damage. Another significant difference between cell cultures and in vivo tissue is that damage in primary cultures following the 30-45 minutes exposures is completely dependent on activation of NMDA receptors (Goldberg P., 1993). Although damage in cultures is easily studied, it is essential to verify any inferences by studies of in vivo tissue. Neuronal gene expression is likely to alter very significantly in culture; the anatomical relationships between cells are very different, and there are no vascular cells. An important technical issue in producing reliable data on protective effects of drugs is a carefully and prolonged temperature control, for at least 24-48 h, after the insult.

Ischemic cell death

Ischemic cell death initially arises from changes that result from inhibition of oxidative phosphorylation including: decreased pH and ATP, free radicals production by mitochondrial chain, increase in Na^+ cellular concentration and membrane depolarisation. These damaging process are characterized by the ability to produce long-term cellular changes and are considered a key step for necrotic cell death after an ischemic insult. Consequently, major

causes of ischemic cell death are generation of ROS, alterations in Ca^{2+} regulation and excitotoxicity.

The generation of reactive oxygen species is a major cause of cell damage following ischemia, in which glutathione (GSH) plays a central role. In ischemia, the role of GSH as oxidant scavenging arising considering its decline during the reperfusion period, when ROS are reported to be largely produced (Shivakumar BR., 1995). In in vitro models, the actions of ROS-generating excitotoxins have been shown to depend on the intracellular GSH level (Ceccon M., 2000), and extracellular GSH has been shown to be neuroprotective both in vivo and in vitro (Cho IH., 2003). Clinical and experimental studies, have provided data supporting the involvement of alterations in Ca^{2+} regulation in the pathogenesis of stroke. Evidence that perturbed cellular Ca^{2+} homeostasis is pivotal to the death of neurons following a stroke is strong. Neurons in the affected brain regions exhibit increased activities of Ca^{2+}-dependent proteases, and inhibitors of calpains protect neurons against ischemic injury in animal models of stroke (Hong SC., 1994). Studies of animal and cell culture models have clearly demonstrated that neuronal Ca^{2+} overload occurs in neurons subjected to ischemia (Kristian T., 1998).

As extensively reviewed by Lipton (Lipton P., 1999), it seems that there are three cell death ways following an ischemic insult: apoptosis, autophagy and necrosis. The last one can be an ischemic/homogenizing cell death (the cell shrinks and become very electron dense), an edematous cell death (the cells swells and organelles loose their structure) or ischemic cell death (the cell shrinks and the nucleolus assumes a honeycomb structure). However, it is not completely known why a specific death way occurs because of a particular ischemic insult. Apparently, one insult can spawn more than one death way in the same cell population. A large number of experimental

observations are supporting the involvement of apoptotic cell death during ischemia, in different cell type and conditions. Evidence are reported for characteristic morphological changes like formation of spherical-shaped mass of chromatin within the nucleus (apoptotic bodies) (Li Y., 1995; Linnik MD., 1993). Apoptotic neurons appear quite soon after a focal insult, and the ratio between apoptotic to necrotic cells can reach 9:1 (Charriaut-Maralangue C., 1996). From a biochemical point of view there are the breakdown of DNA into nucleosomal segments, with fragments multiples of 200bp (DNA laddering), the activation of caspase-3, the inhibition of cell death by inhibition of caspases. Several studies have point out caspase involvement after one or more hours of focal ischemia, showing that there is an earlier increase in the activation of procaspase-3 by cleavage (Sugawara T., 2004).

1.4 Chronic neurodegenerative disorders

1.4.1 Parkinson's Disease

Parkinson's Disease is a progressive and chronic neurodegenerative disorder characterized by hypokinesia, rigidity, tremor and depression associated to α-synuclein protein aggregates (Lewy bodies). The anatomical areas whose degeneration is the cause of PD symptoms are called basal ganglia, a nervous circuit functionally interposed between the cortex and the thalamus. The main task of basal ganglia is to modulate movement execution, processing signals incoming from the cortex, and producing an output that returns to the cortex through the thalamus. Basal ganglia are composed of several nuclei of gray matter (Figure 5A):

I. Caudate nucleus

II. Putamen

III. Globus pallidus, external and internal segment (GPe and GPi)

IV. Substantia Nigra pars reticulata and pars compacta (SNr and SNc)

V. Subthalamic nucleus (STn)

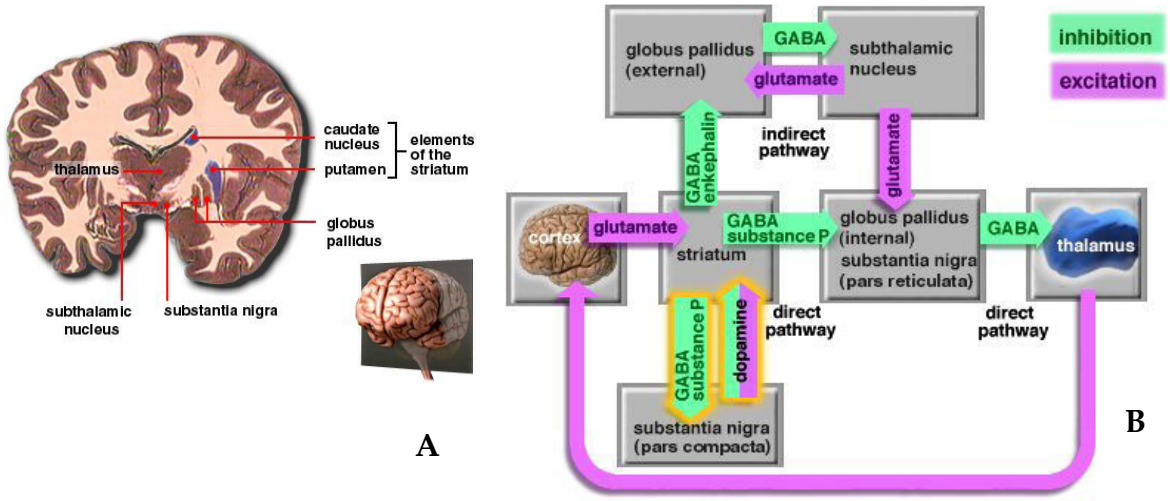


Fig. 5: A) Transversal section of CNS highlighting basal ganglia nuclei. B) Scheme of basal ganglia circuitry functional organization with the indication of direct and indirect pathways. Different colours of arrows indicate different neurotransmitters (glutamate: glu, GABAergic neurons enkephaline positive: GABAenkephaline, GABAergic neurons substance P positive: GABA substanceP).

Although the caudate nucleus and putamen are partly separated by the internal capsule, several bridges of cells connect the two nuclei, which are similar in terms of anatomical and functional characteristics. Therefore, the two structures together are generally referred to as *corpus striatum.* Prevalent neuronal population in the striatum is represented by spiny neurons (95%) that use γ-amino-butyric acid (GABA) as neurotransmitter. The remaining 5% of neurons are aspiny neurons containing alternatively acetylcholine, somatostatine or GABA.

Direct and indirect pathway of basal ganglia

The striatum, the main input of the circuit, transmits the information received from the cortex to the substantia nigra and globus pallidus, via a direct and an indirect pathway. The two pathways originate from different subsets of striatal neurons that remain functionally segregated (Figure 5B):

I. Direct pathway, excitatory monosynaptic way: Striatal GABAergic neurons containing enkephalin project to the SNr and GPe

II. Indirect pathway, inhibitory trisynaptic way: A different subset of GABAergic neurons containing substance P/dynorphin project to the GPi which send projections to the STn

From the output nuclei, inhibitory GABAergic projections reach the thalamus. Thalamic nuclei then send glutamatergic projections to the motor cortex closing the loop. The functional consequence of such organization is that activation of the direct or indirect pathway leads to opposite changes in the output of the basal ganglia circuitry: activation of the direct pathway causes inhibition of GABAergic neurons leading to disinhibition of thalamic nuclei and consequent activation of motor activity. On the other hand, activation of the indirect pathway causes inhibition of the GPi. The subsequent activation of the glutamatergic fibers of subthalamic nucleus induces an enhancement of the inhibitory control over the motor activity (Alexander GE., 1990).

Neurodegeneration in PD

The striatum is the main input of the basal ganglia, and the major input to the striatum is of excitatory nature. In fact, glutamatergic projections from all cortical areas, thalamus and some limbic structures converge onto striatal neurons. Another essential input to the striatum is represented by dopaminergic inputs incoming from SNc and the ventral tegmental area (VTA). Dopaminergic inputs can be distinguished between two systems:

I. Nigrostriatal system

II. Mesolimbocortical system

The nigrostriatal system projects from the SNc to Striatum (the Caudate - Putamen) and the mesolimbocortical system, projects from the ventral tegmental area to the nucleus accumbens, olfactory tubercle, prefrontal cortex and amygdale (Dahlströrm A., 1964). The mesostriatal system is essentially involved in motor control whereas the mesolimbocortical system is more important for reinforcing and reward-related behaviours (Nestler EJ., 2005) (Figure 6). The main targets of striatal projections are the medial and lateral segments of the globus pallidus and the substantia nigra pars reticulata.

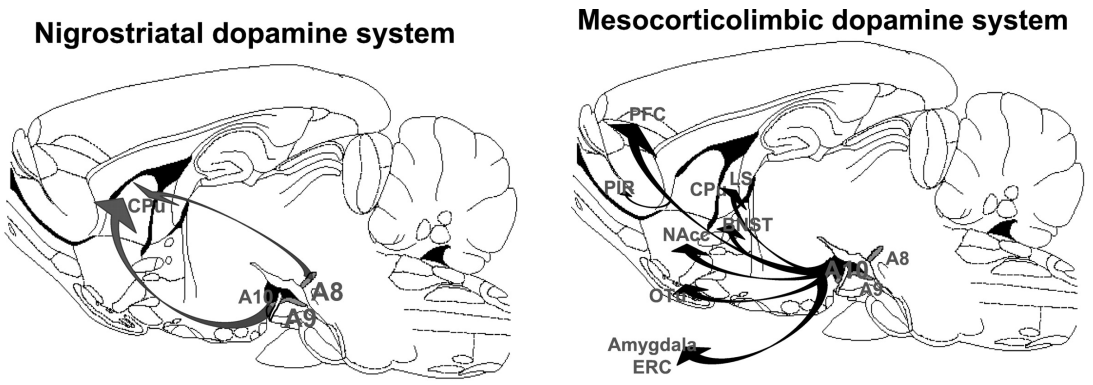


Fig. 6: Origin and major projections of the mesocorticolimbic and nigrostriatal DA pathways. There are three DA midbrain nuclei: the SNc and SNl (A9), the VTA (A10), and the RRF (A8). These neurons give rise to the nigrostriatal and mesocorticolimbic DA projection systems. SRl: Substantia Nigra pars lateralis; RRF: Retrotubal fields; PIR: piriform cortex; Otu: olfactory tubercles (Adapted from: Binder EB.,2001)

Glutamate plays a pivotal role in the regulation of striatal activity. The caudate nucleus shows the highest density of glutamate receptors in the basal ganglia circuitry, reflecting the abundance of glutamatergic projections that reach the striatum, particularly from the cortex, with a preferential expression of NR1, NR2B and NR2C subunits of NMDA receptor in the

human striatum, compared to the globus pallidus (Bernard V., 1998; Kosinski CM., 1998).

The pathological hallmarks of PD are the progressive formation of Lewy bodies and the loss of the nigrostriatal dopaminergic neurons. The degeneration of that neuron, which normally contains conspicuous amounts of neuromelanin, produces the classic gross neuropathological finding of SNc depigmentation (Figure 7).

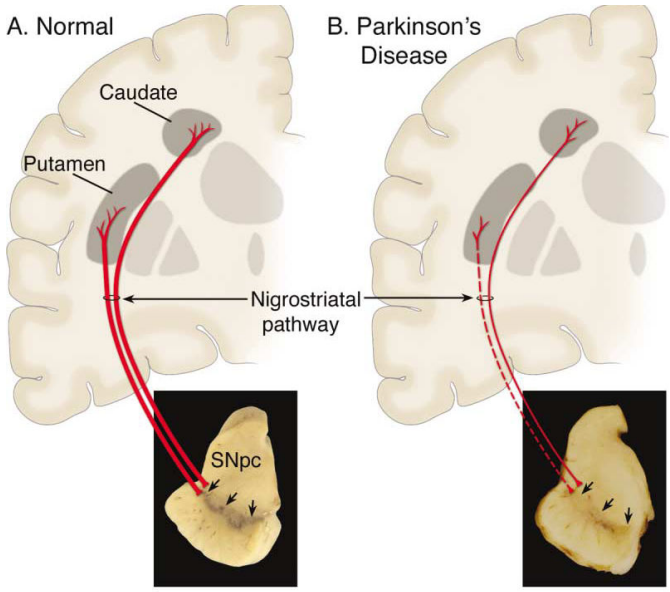


Fig. 7: (A) Schematic representation of the normal nigrostriatal pathway (red). It is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc). These neurons project (thick solid red lines) to the basal ganglia and form synapse in the striatum (i.e., putamen and caudate nucleus). The photograph demonstrates the normal pigmentation of the SNpc, produced by neuromelanin within the dopaminergic neurons. (B) Schematic representation of the diseased nigrostriatal pathway (red). In PD the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). The photograph demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin) of the SNpc due to the marked loss of dopaminergic neurons. (Adapted from: Dauer W., 2003).

The degeneration of mesostriatal dopaminergic neurons arise from several causes including genetic predisposition, environmental noxe and aging. Pathological features of PD are: mitochondrial dysfunction, oxidative

stress, excitotoxicity, inflammation and proteasome failure (Onyango IG., 2008; Dauer W., 2003).

As saying, excitotoxicity can be the direct result of excessive stimulation of the NMDA receptor, due to increased levels, or decreased removal from the synaptic cleft, of glutamate. A direct action of glutamate is likely to play a role in acute neurological disorders, such as hypoxic/ischemic brain damage, but not in a chronic disorder, such as PD. In fact, large increases in extracellular glutamate occur in the hypoxic/ischemic damage (Rothman SM., 1986), but not in PD.

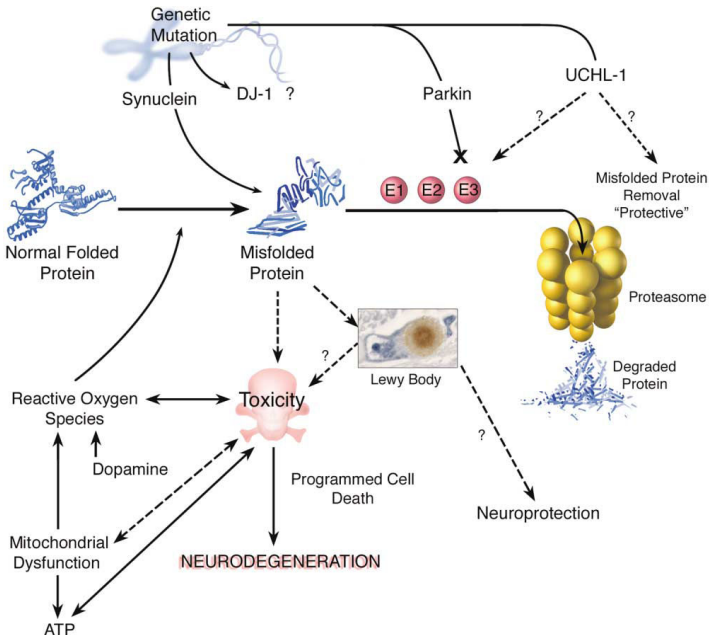


Fig. 8: The diagram illustrates the relationship that may link the various mechanisms potentially involved in the pathogenesis of PD. Such mechanisms include mitochondrial or genetics defects, excitotoxicity, excessive free radical formation and depletion of endogenous anti-oxidants, can be viewed as parts of a cycle, in which any one of these processes might lead to all of the others. (Adapted from: Dauer W., 2003).

However, glutamate can be toxic indirectly, because if a neuron is depolarized the Mg^{2+} blockade is relieved and binding of glutamate leads to a large Ca^{2+} influx. Maintenance of membrane polarity is a process that requires continuous energy supplementation so, impaired mitochondrial function causes depolarization (Erecinska M., 1990) and the result is that

physiological levels of glutamate become toxic. Substantial experimental evidence has supported this hypothesis, showing that the inhibition of mitochondrial respiration, both in vitro and in vivo models, causes excitotoxic lesions. The excitotoxic nature of these lesions is confirmed by the fact that they are prevented by NMDA antagonists (Blandini F., 2000). Mitochondrial abnormalities have been reported repeatedly in the substantia nigra pars compacta of PD patients. Therefore, a synergistic interaction between bioenergetic deficits and glutamatergic stimulation is likely to play a foundamental role in the degeneration of nigral neurons (Figure 8).

Animal models of PD

There are several in vitro and in vivo models for PD:

I. Toxin-based models: farmacological models

a. Lesion of the nigrostriatal pathway, based on the intracerebral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) (Ungerstedt U., 1968). 6-OHDA affects mitochondria by inhibiting complex I and inducing oxidative stress. This model is chracterized by wide dopamine degeneration, contralaterally rotation following administration of dopaminergic antagonists or precursor like apomorphine and L-DOPA, absence of Lewy bodies inclusion. The study of new treatments for PD, including gene terapy, has prevalently evaluated in this model using the contralateral rotation effect as experimental tool.

b. Intra-carotid injection of the neurotoxin MPTP that causes selective degeneration of dopaminergic neurons of SNc resulting in PD-like sindrome (DeLong MR., 1990), without Lewy bodies inclusion.

c. Chronic treatment with Rotenone, a strong inhibitor of mitochondrial complex I, known to be involved in PD etiology. Rotenone induces nigrostriatal neurodegenration, reduction of Tyrosine hydroxilase and dopamine transporter expression

II. Gene-based models: transgenic mice

a. Two missense mutations in α-synucleine encoding gene cause a dominantly inherited PD (Polymeropoulos MH., 1997; Kruger R., 1998**).**

b. Loss-of-functions mutations in the parkin encoding gene cause a recessively inherited parkinsonism (Kitada T., 1998)

1.4.2 Dopaminergic System

The dopaminergic system activity is essential for movement contol, modulation of behaviour, cognitive functions and reward/addiction mechanisms. Dopamine (DA) is a cathecholamine, synthetyzed by the enzyme tyrosine hydroxylase (TH) from the aminoacid Tyrosine. TH immunoreactivity ia a widely used marker of DA neurons and the localization of TH(+) immunoractive cells allows identification of DAergic projections. After its sythesis, DA is stored in synaptic vesicles and released in a Ca^{2+}-dependent manner and signal transduction is terminated by reuptake of DA into the presynaptic terminal by the DA transporter.

DA neurotransmission involve five different receptors type, subdivided into two groups based on the mechanism of cAMP signalling regulation:

I. D_1 - like: D_1 and D_5: stimulation of adenylyl cyclase

II. D_2 - like: D_2, D_3 and D_4 : inhibition of adenylyl cyclase

Dopaminergic receptors are expressed both presynaptically (autoreceptor) and postsynaptically. Autoreceptor activation inhibits DA transmission and

TH synthesis in DA neurons. Is important to note that DAergic synapses seems to be "open" synapses, allowing DA diffusion into extracellular space and consequent activation of dopaminergic receptors distant from the release site in a paracrine way.

D_1 and D_2 receptors types are higly expressed in striatum, with specifc subcellular distribution. D_2 are found on dopaminergic nerve terminals and postsynaptically on GABAergic medium spiny neurons (the 95% of the striatal neurons). GABAergic medium spiny neurons can be divided into two sub-populations: those terminating in SNr (striatonigral neurons) and those terminanting in the GPe (striatopallidal neurons). Electrophysiological studies suggest that dopaminergic transmission modulate the striatal responses to incoming inputs, particularly those mediated by glutamate (Calabresi P., 1997). Release of glutamate in the striatum seems to be modulated, in part, by nigrostriatal dopaminergic projections so, chronic blockade of D_2 dopamine receptors can cause an increase in the levels of both basal extracellular and potassium-released glutamate in striatum.

A modulator that influences the responses of dopamine receptors in the striatum is adenosine. Adenosine acts on specific receptors (A_1, A_{2A}, A_{2B} and A_3) that appear to be co-localized with dopamine receptors. In particular, A_1 receptors are colocalized with D_1 receptors, while A_{2A} receptors colocalize with D_2 receptors. In both cases, adenosine antagonizes the effects mediated by dopamine on striatal neurons. Stimulation of A_{2A} receptors inhibits the release of GABA from the globus pallidus following the activation of striatal D_2 receptors (Ferré S., 1993; 1996).

Receptor heterodimers

It was in the earlier 1980th that a new concept of receptor activity and modulation arise, the intramembrane receptor-receptor interaction and cooperation, leading to the introduction of the receptor mosaic hypothesis

(Fuxe K., 1983; 2007). It was postulated that clusters of receptors could be formed via structural receptor-receptor interactions in the postsynaptic membrane, as a consequence of the synaptic activity itself.

The formation of receptor heterodimers could happen by three distinct mechanisms:

I. Direct interaction at the membrane level between two receptor molecules

II. Indirect interaction between receptors by a "scaffold" protein

III. Indirect interaction between receptors by an "adapter" protein that induce receptor conformational changes

The subsequent formation of receptor mosaic (RM) structures could be of three different types: RM1, homoligomers, built up of the same type or subtypes of the same receptor; RM2: heteroligomers, built up of different type of receptors; mixed RM: built up of RM2 with inclusion of islands of RM1.

The existence of a functional antagonistic A_{2A}/D_2 heterodimer located on dendritic spines in the synaptic areas of DAergic and glutamatergic terminals in the striatopallidal GABA neurons arise from several experimental studies (Ferré 1991; 1994; 1997; Tanganelli S., 2004; Fuxe K., 2007). In consideration of that modulatory activity of A_{2A} recptor on dopaminergic transmission, an increasing number of experimental studies has been done in view of a possible role for A_{2A} antagonists in the treatment of PD. In an in vivo PD model A_{2A} antagonists are found to increase, in a dose-dependent way, locomotor activity with threshold doses of levodopa and D_2 antagonists (Fuxe K., 2008).

2. NEUROTENSIN SYSTEM

Since its discover in the earlier 1970th by Carraway and Leeman (Carraway R., 1973), the tridecapeptide neurotensin (NT, Figure 9) was studied for its neuromodulatory property.

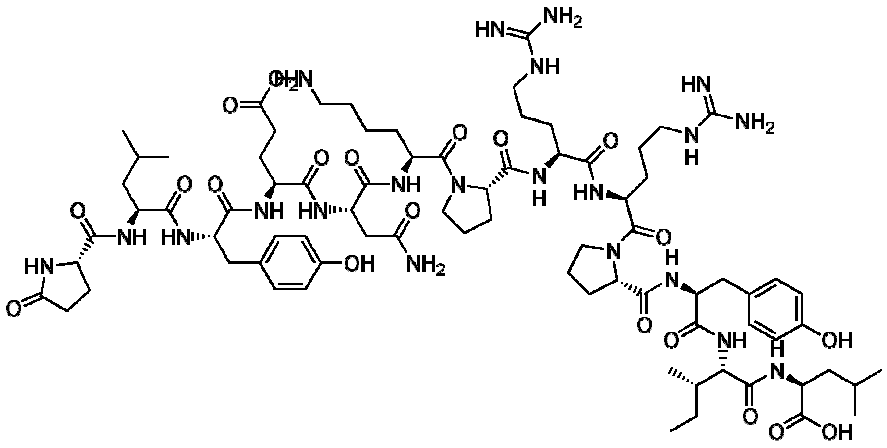


Fig. 9: Structure of the tridecapeptide neurotensin (NT).

Neurotensin exerts potent central effects including hypothermia, antinociception, modulation of neurotransmission and stimulation of anterior pituitary hormone. Peripherally, neurotensin acts as a paracrine and endocrine hormone for the digestive and cardiovascular systems. Like many other neuropeptides, NT can act as neurotransmitter or modulator of classical neurotransmitter like dopamine and glutamate. In consideration of that neuromodulatory ability, as been suggested that the neurotensinergic system might be implicated in several CNS diseases, consequents DAergic and glutamatergic disfunctions, like schizophrenia, drug abuse, PD as well as more recently in cerebral ischemia (Antonelli T., 2008).

2.1 Neurotensin system

NT is synthesized as part of a larger inactive precursor, pre-proneurotensin (pre-proNT/NN), located on human chromosome 12, that also contains neuromedin N (NN), a 6-aminoacid NT-related peptide (Hayes RG., 1992). In the rat, the NT/NN precursor consists of 169 amino acid residues, The four amino acids at the carboxy terminal of NT and NN are identical, and amino acids 8-13 of NT are essential for biological activity (de Nadai F., 1994). Once processed as an active peptide by endopeptidases belonging to the family of pro-protein convertase (PCs), NT is stored in dense core vesicles and released in a calcium-dependent way.

NT-expressing neurons are widely distributed in the CNS, explaining the wide range of effects of this peptide. NT mRNA is highly expressed in hypothalamus, amygdala, nucleo accumbens, caudate-putamen and cerebral cortex (Alexander MJ., 1989). The strong expression of NT in DAergic areas (Figure 9) as basal ganglia, can explain the experimental evidences that suggest an essential involvement of NT in the neuromodulation of DA release (Rostene W., 1992).

Upon the above consideration, a putative role of NT the development of PD has been suggested from several authors. Clinical post-mortem binding data obtained from brains of PD patients indicates that NTS1 protein (Uhl GR., 1984; Chinaglia G., 1990) activity and NT expression results altered in basal ganglia (Fernandez A., 1994). Nevertheless, actually the exact mechanisms by which NT potentially induces neurodegeneration remain unclear, with only few studies that correlate NTS1 activity to an amplification of the excitotoxicity-induced neurodegeneration mechanism (Antonelli T., 2002; 2004; Tomasini MC., 2005).

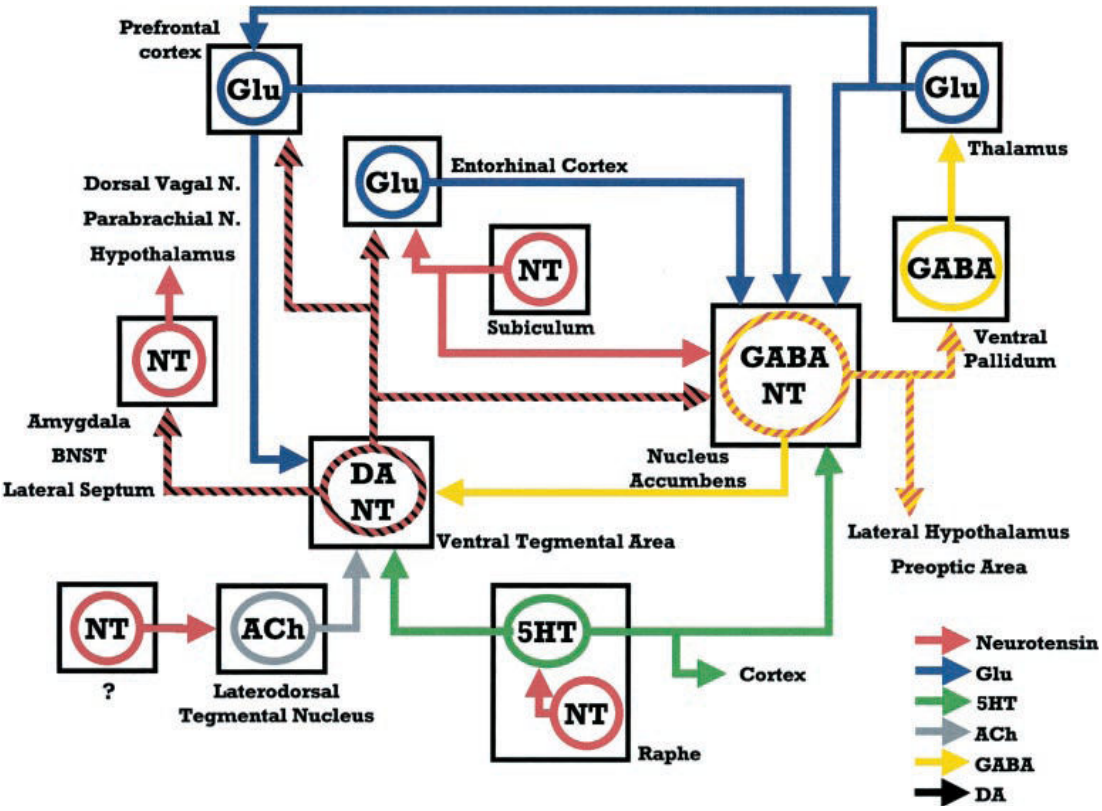


Fig. 9: Summary of NT system interactions within mesocorticolimbic DA system. (5HT: Serotonin receptor , Ach: Acetylcholine) (Adapted from Binder EB., 2001).

Neurotensin receptors

NT central and peripheral activities are consequences of NT receptors activation. Three different NT receptors, called NTS1, NTS2 and NTS3/sortilin, have been cloned to date. NTS1 and NTS2 belong to the G-protein coupled receptor family, with the typical 7-transmembrane α-elix structure. A candidate fourth NT receptor has been proposed, although little is actually known about its physiological relevance. This receptor, called SorLA/LR11, like NTS3 is a single transmembrane domain receptor type 1 (St.Gelais F., 2006). In the CNS this receptor is expressed in neurons, but its role in NT signalling effects remains to be clarified.

Type 1 NT receptor is often referred to as the high-affinity binding for NT site (Kd = 0.1-0.3 nmol/L). NTS1 was originally cloned in the rat and is

encoded on human chromosome 20. In rat brain, NTS1 transcripts are located primarily in neurons of the hypothalamus, septum, amygdala, substantia nigra, VTA, nucleus accumbens, zona incerta, suprachiasmatic nucleus, prefrontal, entorhinal and retrosplenial cortices (Binder EB., 2001).

NTS1 is functionally coupled to the phospholipase C, responsible for phosphatidil-inositole 4,5-phosphate idrolisis with production of diacilgricerole (DAG) and the inositol 1,4,5-phosphate (IP3). Such signalling cascade is related to calcium intracellular levels increase, thus inducing excitatory effects.

Other possible signalling pathways could occur through activation of cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), arachidonic acid production,54,55 mitogen-activated protein (MAP) kinase phosphorylation (Rostene W., 1997).

The effects mediated by NTS1 are generally blocked by its selective non peptide antagonist SR48692.

2.2 Neurotensin system modulation of dopaminergic signalling

The discovery that NT is colocalized with DA in mesocortical neurons, suggest that it could play a role in the regulation of mesocortical DA transmission (Hokfelt T., 1984). The complex interactions that exist between NT and DA in the VTA, nucleus accumbens and cerebral cortex include a possible NTS1/D_2 receptor-receptor interaction (Ferraro 2007). Anyway, the functional interaction between NTS1 and D_2 receptors lead to a reduction of D_2 function (Jomphe C., 2006). In this context, a potential antipsychotic profile for NTS1 antagonists has been proposed based on their ability to block postjunctional D_2 transmission in the nucleus accumbens (Caceda R., 2006) without increasing DA release (Tanganelli S., 1994).

In the nucleus accumbens local activation of NTS1 by NT at low (10nM, in vivo microdyalisis technique) nanomolar concentration induces an enhancement of GABA release, in contrast to that observed in the dorsal striatum (Tanganelli S., 1994). The alteration observed in GABA release is associated with a significant reduction in the accumbal DA release. These effects have been proven by the fact that the blockade of $GABA_A$ receptor in NAc by its antagonist bicuculline, counteracts the inhibitory effects of NT on DA release without altering the enhancement of GABA release (Tanganelli S., 1994). NT localization on cortical afferents in the nucleus accumbens, particularly on dopaminergic and glutammatergic axon terminals (Pickel VM., 2001), suggest the idea that NTS1 activation on accumbal glutamate terminals can induces an enhancement of glutamate release. The consequent activation of inhibitory GABAergic signalling of the striato-pallidal pathway could cause a significant reduction of DA release as observed (Tanganelli S., 1994). The ability of NT to induce an enhancement in glutamate release is supported by several in vivo and in vitro data (see next paragraph).

The antagonistic NTS1/D_2 interaction effects at striatum level substantially differs from those in the NAc (Figure 10). In fact intrastriatal perfusion with D_2 agonist pergolide inhibits both DA and GABA release, whereas the co-perfusion with a low ineffective nanomolar concentration of NT show a complete recovery of DA and GABA extracellular levels. This NT effect results completely counteracted by the presence of the NTS1 antagonist SR48692 (Gully D., 1993).

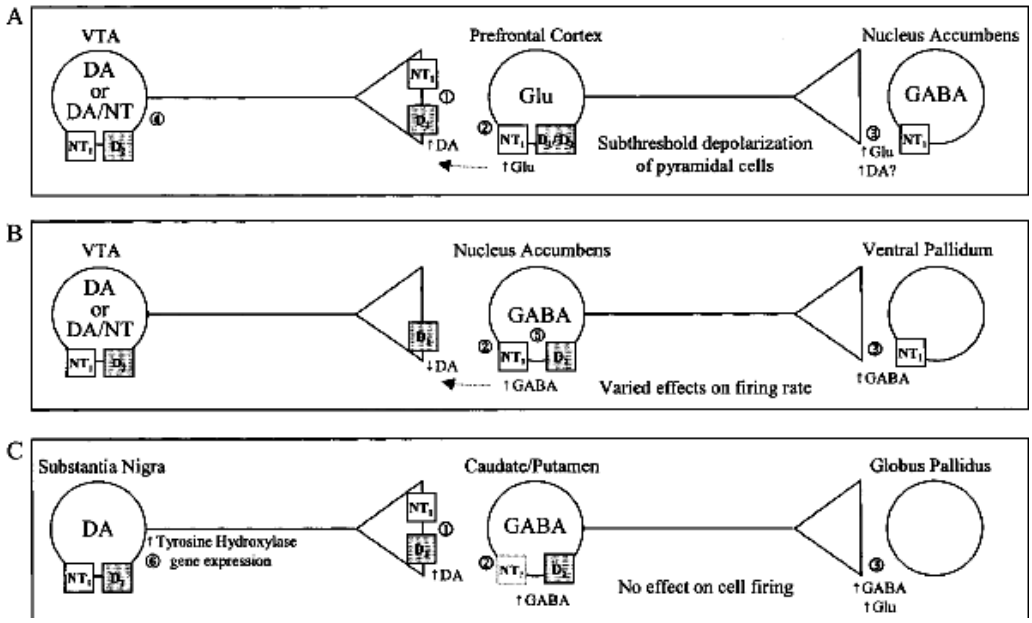


Fig. 10: Summary of the electrophysiologic and neurochemical effects of NT administered directly into the prefrontal cortex, nucleus accumbens, or caudate/putamen. A) Prefrontal cortex: NT administered directly into the prefrontal cortex increases DA release via a presynaptic decrease in DA autoinhibition (1) and increases glutamate release via a direct postsynaptic effect (2). NT also blocks the inhibitory effects of DA on prefrontal cortical cell firing, leading to an increase in glutamate release and a consequent increase in DA release in terminal regions of the prefrontal pyramidal neurons such as the nucleus accumbens (3). Microinjection of NT into the prefrontal cortex increases the firing rate of DA-ergic neurons in the VTA (4). B) Nucleus accumbens: Low dose NT injected directly into the nucleus accumbens increases GABA release via either a direct postsynaptic effect (2) or a decrease in postsynaptic D2 receptor-mediated inhibition (5) leading to a decrease in DA release via activation of presynaptic GABA receptors. NT stimulation of GABA-ergic neurons in the nucleus accumbens through direct stimulation and decreased D2 receptor-mediated inhibition, leading to an increase in GABA release in accumbal terminal areas such as the ventral pallidum (3). C) Caudate/putamen (Striatum): NT injected directly into the CPu increases the release of DA by decreasing presynaptic D2 autoinhibition (1). Interestingly, GABA release is also increased and this effect has been attributed to a novel postsynaptic NTR (2). GABA release is also increased in the projection area of D2 positive GABA-ergic neurons (3), indicating that intra-CPu administration of NT stimulated striatopallidal neurons. (Adapted from: Binder EB., 2001).

The NTS1/D_2 receptor-receptor interaction hypothesis is supported by receptor binding and autoradiographic studies (von Euler G., 1990) and this

modulation mechanism is observed in limbic brain regions as well as in the striatum (Tanganelli S., 1993). The functional evidence that, in NAc, the antagonistic NT/DA interaction take places primarily at postjunctional level, arise from in vivo microdyalisis experiments by Li XM. & coll. (Li XM., 1995). They have demonstrated how administration of intra-accumbal low nanomolar concentration (1nM) of NT by itself ineffective on DA and GABA extracellular levels, if co-perfused with the D_2 agonist pergolide, can significantly counteract the inhibitory effect of the D_2 agonist on GABA release without affecting the inhibition of DA release (Figure 11).

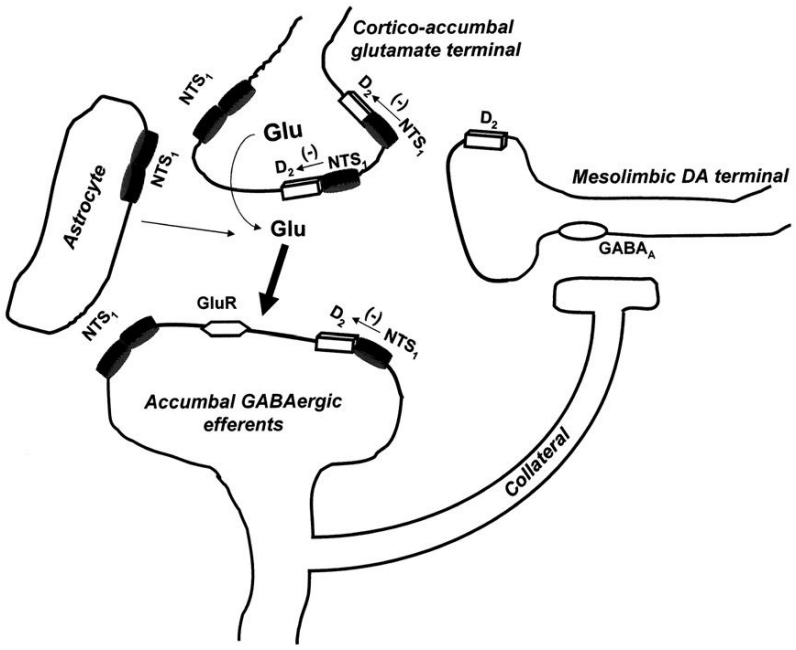


Fig. 11 : Main location of the NTS1/D_2 heteromers mediating the NTS1/D_2 receptor–receptor interactions in the control of the ventral striato-pallidal GABA pathway from the nucleus accumbens. In the nucleus accumbens there exists a dominance of the postjunctional antagonistic NTS1/D_2 receptor–receptor interactions on the cortico-accumbens glutamate terminals over the weak antagonistic NTS1/D_2 receptor–receptor interaction on the accumbens DA terminals (not shown). The intra-accumbens perfusion of NT antagonizes the inhibitory dopamine D2 tone on glutamatergic terminals via a NTS1/D_2 receptor–receptor interaction thus inducing an enhancement of glutamate outflow. Such an increase can then activate the inhibitory GABAergic signalling that could be responsible, via a collateral, for the significant reduction of DA release observed in the nucleus accumbens (Tanganelli et al., 1994). However, it cannot be excluded a direct involvement of NTS1 located on GABAergic neurons (Binder et al., 2001). The two D_2/NTS1 heteromers on the glutamate terminals are to show that this is their major location, since only few are found on the striato-pallidal GABA neurons. NTS1 alone without D_2 could be a monomer or a homomer which is true also for

D2 when not present in a heteromer. Finally, although there is no direct evidence today for NTS1 modulating astroglia glutamate release, the possibility for astrocytes as part of the neurochemical substrates that give rise to the release of glutamate is also represented. (From Ferraro L., 2007, Paper I).

These data further support the existence of an antagonistic NTS1/D_2 interaction at cortico-striatal glutamate and striatal DAergic terminals level. In contrast, in the NAc, the same antagonistic interaction takes place on cortico-accumbens glutamate terminals and at postsynaptic level of efferent GABAergic neurons. NT induces a substantial increase effect on glutamate thus, release at both striatal and accumbal level that counteract the dopaminergic D_2 inhibition of GABAergic signalling.

In view of the above consideration, the intimate role of mesolimbic and mesocortical DA disfunctions in the eziopathogenesis of schizophrenia and psychomotor stimulant/reinforcing drug effects open up a potential role for NT signalling (Kinkead B., 2002; 2006**;** Caceda R., 2006).

2.2.1 Relevance in schizophrenia and reward behaviour

Schizophrenia

Concerning the pathopysiology of schizophrenia it seems that a primary hyperactivity of the mesolimbic DA system and hypoactivity of mesocortical DA system (Carlsson A., 1999) could be a primary neurochemical cause. In view of the close interaction between NT and DA system at mesolimbic level, and of the relevance for these pathways in schizophrenia eziopahtogenesisi, Nemeroff proposed a potential antipsychotic role for NT (Nemeroff CB., 1985**;** Binder EB., 2001**;** Caceda R., 2006). Briefly, potentially NT antipsychotic effect could be a consequence of its ability to induce an increase in striato-pallidal GABAergic transmission. Consequently, the NTS1/D_2 antagonistic interaction (see above) can restore the excitatory pathway of thalamo-cortical glutamate projection apparently reduced in schizophrenic patients.

Reward behaviour

A neurochemical essential feature in NT-dependent reward mechanism is that psychostimulants drugs can increase NT release in specific striato-nigral GABAergic neurons involved in the reward phenomenon (Merchant KM., 1994) that are essentially modulated by D_1 receptor activity. These receptors are not modulated by an antagonistic interaction with NTS1. The DA increase, indirect consequence of NTS1/D_2 interaction (see above), is associated with D_1 receptor activation increase favouring these reward pathways. A second possible mechanism, involve the ability of NT to activate NTS1 receptor on glutamatergic teminals of the same circuitry. The consequent glutamate release can activate specific striato-nigral and accumbal-VTA GABAergic pathways intimately involved in psychostimulant reward pathways (Ferraro L., 2007, paper I).

2.3 Neurotensin system modulation of glutammatergic signalling

As mentioned above, striatal NT receptor modulates striatal and limbic GABAergic neuron activity trough an antagonistic NTS1/D_2 interaction, leading to an enhancement of dopaminergic transmission via D_1 receptor activation and consequent motor inhibition via thalamocortical circuitry (Antonelli T., 2007). Consequently, it could be hypotezised that the NTS1/D_2 heteromeric complex expressed at striatal glutamatergic terminals, by antagonizing the D_2 inhibitory function can lead to an increase in glutamate release. Anyway, the ability of NT to induce an enhancement of glutamate transmission by a facilitatory NTS1/NMDA interaction is supported by several in vivo and in vitro data (Ferraro L., 1998; 2000; 2001).

As reported in figure 12, NT has been shown the ability to induce, in vivo, an increase of glutamate transmission at both corticostriatal and subthalamic

level, in a concentration-dependent way (Ferraro L. 1998). Furthermore, the authors, by performing further in vivo microdyalisis experiments with the NTS1 antagonist SR48692, were able to ascribe such effect at the specific activity of neurotensin receptor type 1.

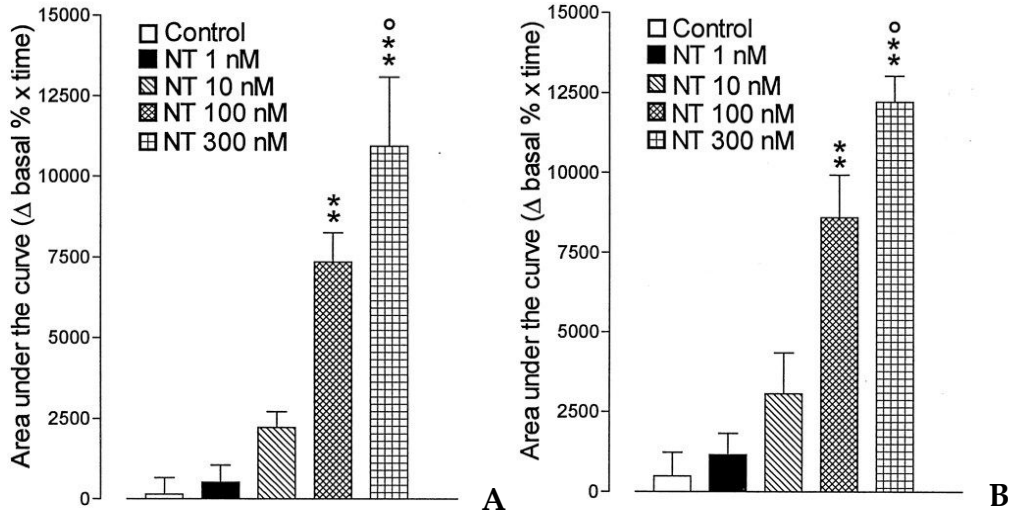


Fig. 12 : Effects of intrastriatal perfusion with NT on striatal (*A*) and pallidal (*B*) glutamate release in the awake rat. The histograms represent the areas under the curves of classical microdyalisis experiment, calculated as percentage of changes in basal value over time. ** p < 0,01 significantly different from control gropu; ° p < 0,05 significantly different from NT 100nM group. Adapted from Ferraro L., 1998)

Besides, NT is also able to induce an amplification in glutamate extracellular release at cortical level with a bell-shaped dose-response curve (Figure 13), as evdenced by recent in vitro experiment on rat cortical slices and cultured neurons. Also in this case, the neurotensinergic-induced glutamate release seems to be a direct consequence of NTS1 receptor activation (Ferraro L. 2000; Antonelli T., 2004). Moreover it seems that the cross talk between NT and glutamate transmission could be mutual (Radke JM., 2001), with a specific involvement of the NMDA gultamatergic receptor, even if further

studies are necessary to clarify the exact role of NMDA receptor between those mechanisms.

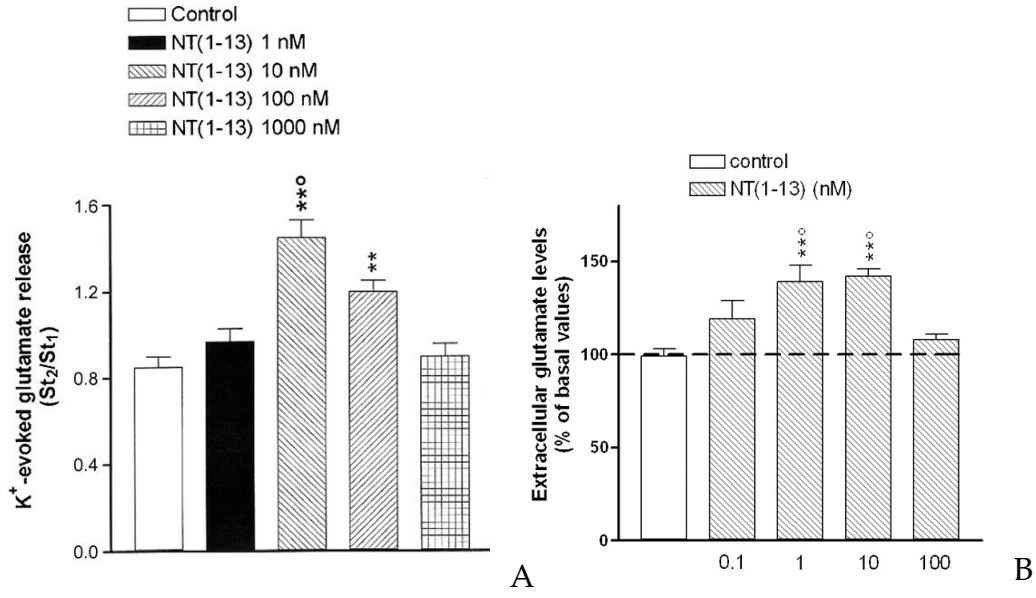


Fig. 13 : Effects of neurotensin NT(aminoacids 1–13) on K^+ (35 mM)-evoked endogenous glutamate release from rat cerebral cortex slices (A) and extracellular glutamate levels in primary cultures of rat cerebral cortex neurons (B).
A) The data are presented as histograms of the St2/St1 ratio. K+-evoked glutamate release were expressed as percent increase over the spontaneous (i.e. basal) glutamate release, as calculated by the mean of the two fractions collected prior to the depolarising stimulus. ** $p < 0,01$ significantly different from control, NT 1nM and NT 1000nM groups; ° $p < 0,05$ significantly different from NT 100nM group. (Adapted from Ferraro L., 2000).
B) Glutamate levels measured in the third fraction from the start of the experiment are reported as percentage of the basal values calculated from the first two fractions. NT was applied at the onset of the third fraction and manteined for 30'. ** $p < 0,01$ significantly different from control group; ° $p < 0,05$ significantly different from NT 0,1nM and NT 100nM groups. (Adapted from Antonelli T., 2004).

Recently, electrophysiological evidences obtained from dorsolateral striatum of rat has show that NT seems able to inhibits glutamate transmission reducing presynaptic glutamate release, but only at higher concentrations (500-1000nM) (Yin HH., 2008). Such effect, apparently incoherent with the previous findings, require the induction of an endocannabinoid inhibitory retrograde signalling (Yin HH., 2008). Therefore, it is possible that, in a first step, NT can induce an increase in glutamatergic transmission, causing the

activation of postsynaptic metabotropic glutamate receptors (mGluRs), notoriously involved in cannabinoid signalling (Giuffrida A., 1999; Yin HH., 2006). In fact, experimental in vivo evidences has strongly implicated the cannabinoid signalling in the regulation of glutamatergic transmission at corticostriatal level, showing that inhibition of CB1 cannabinoid receptor can lead to an enhancement of glutamate release in a in vivo model of PD (García-Arencibia M., 2008).

As reported in figure 13B, in vitro experiment on rat cortical primary cell cultures have shown that NT (1-10 nM) induces a significant increase in extracellular basal glutamate level via NTS1 activation, as evidenced by the use of NTS1 antagonist SR48692 (Antonelli T., 2004). These in vitro experiments, has highligthted for the first time that the link between NTS1 and glutamate modulation receptor seems to involve a NTS1/NMDA interaction via a Ca^{2+} -dependent mechanism. In fact, as suggested by further in vitro experiments of Antonelli & coll., the NT modulatory activity on glutamatergic release previolusy reported results totally absent when NT is administrated in a low-calcium medium (Antonelli T., 2004).

Because of the ability of NTS1 receptor to interact with others receptors type as the dopaminergic D_2, has been suggested that the NT modulation of glutamatergic signalling could rise from a NTS1/NMDA receptor-receptor interaction, nevertheless the possibility of a cytoplasmic interaction between the two receptors remains. Anyway, substantial experimental evidence of a close facilitatory interaction between those receptors has been obtained (Tomasini MC., 2005).

2.3.1 Relevance in neurodegeneration

In view of the critical role played by glutamatergic transmission in several neurodegenerative disorders, the ability of NT to induce an amplification of NMDA signalling takes a strong significance. Morphological, biochemical and immunocytochemical data obtained in in vitro models of excitotoxicity supporting the hypothesis that, the NT-induced amplification of glutamatergic transmission, is implicated in the neuronal cell death during degenerative processes. In fact, it seems that an enhancement of cell death is driven by NTS1 activation at both mesolimbic (Antonelli T., 2002) and cortical levels (Antonelli T., 2004) following a mild excitotoxic insult. In vitro experiments performed on mesencephalic-cultured neurons (Figure 14), that express functional NT and glutamate receptors on dopaminergic cells, has show a loss in dopaminergic function when exposed to a low glutamate insult (30μM), as evidenced by the reduction in [^{3}H]-DA uptake analysis. Such reduction of neuronal physiological activity results sharpened by the co-exposure to a low or medium concentration of NT (Antonelli T., 2002). In accordance with previous finding reported, also in this case the use of SR48692 allows to ascribe such neurotensinergic effect at the activation of its receptor NTS1.

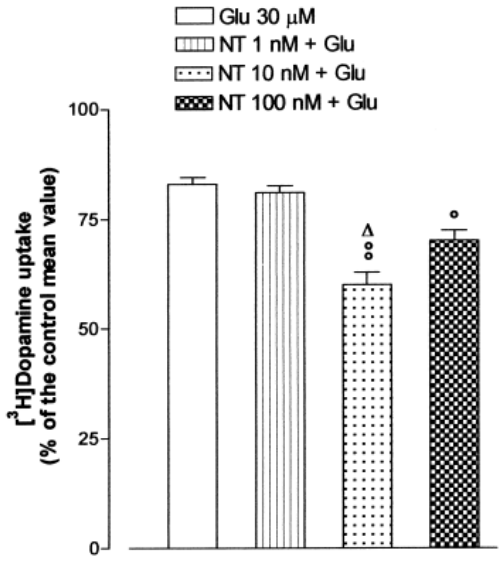


Fig. 14 : Effects of neurotensin (NT) on glutamate (Glu) 30 μM-induced reduction of [^{3}H]DA uptake in cultured mesencephalic cells. The cultures were exposed to 30 μM Glu for 10 min, and NT was added 50 min prior to Glu and maintained in contact with the cells during the Glu exposure (total treatment time 60 min). [^{3}H]DA uptake was assessed 24 hr later. The results are expressed as percentage of [^{3}H]DA uptake measured in cultures not exposed to Glu. °$p < 0.05$, °°$p < 0.01$ significantly different from Glu alone as well as from NT (1 nM)- plus Glu-treated group. $^{\Delta}p < 0.05$ significantly different from NT (100 nM)- plus Glu-treated group. (Adapted from Antonelli T., 2002).

The involvement of NMDA receptors in the hypotesized NT-amplification of glutamate excitotoxic mechanisms was evaluated in a series of successive in vitro experiments (Antonelli T., 2004). In this model, the putative NTS1/NMDA interaction was confirmed, highlighting a possible mechanism of interaction between the receptors. The autors suggest that NT, by activating the protein kinase C (PKC), can mediate a potentiation of NMDA receptors activity by phosphorylation of the receptor itself.

PKC, with phospholipase C (PLC) and inositol-3-.phosphate (IP3), represent the principal transduction pathway of NTS1 receptor, and it is notoriously involved in NMDA receptor phosporilation, with specificity for the different NR subtypes. PKC can phosphorilates NR1 subunit, with an increasing specificity depending on the NR2 subunit associated. NR2A and NR2B containing receptors are both potentiated by PKC activation (Lynch DR., 2001, 2002). The highest level of increase for the open probability and

intracellular calcium levels is for the NR1-NR2A containing receptors followed by NR1-NR2B containing receptors (Waxman EA., 2005). Both NR2A and NR2B subunits are closely related to the NMDA-induced excitotoxic effect of glutamate.

On the basis of the above consideration on NT modulation of aminoacidergic signalling in mesolimbic and mesocortical areas, the hypothesis of the involvement of NT signalling in the eziopathogenesis of chronic and acute neurodegenerative disorders has reached an increasing interest, with specific regards to those neurodegenerative diseases that were, at least in part induced by an excitotoxic insult.

2.4 Neurotensin system and Parkinson's Disease

Several, even if conflicting, evidences suggest that NT expression in PD patients and experimental PD models is enhanced (Fernandez 1995; 1996). These pre-clinical and clinical data could be explained considering the NTS1/D_2 interaction at striato-pallidal level. PD is charazterized by a reduced DA innervation and so by a reduced D_2 autoreceptor transmission that leads to an increased A_{2A} signalling. Increment in A_{2A} transmission can induce NT sythesis and release (dendritic level). The last step of this modulatory loop is the increase in D_2 autoreceptor inhibition by NT activation of NTS1/D_2 antagonistic interaction (Antonelli T., 2007) with a consequent compensatory enhancement of dopaminergic transmission. Nevertheless, NTS1 activation could cause energy impairment in DA neurons and an enhancement in glutamate release via NTS1/D_2 interaction, concurring in neurodegeneration. Besides, the suggested facilitatory NTS1/NMDA interaction on nigral glutamatergic terminals could play a foundamental role in enhancing glutamate excitotoxicity and thus neurodegeneration (Doble A., 1999; Przedborski S., 2005) in PD experimental models (Antonelli T., 2007).

AIM

From its discover to date, the neuropeptide neurotensin has shown to have a wide range of neuromodularory properties. Initial studies upon NT role on CNS functions have highlighted its involvement in specific brain areas, with a well-characterized localization of the peptide and its specific receptors.

Quickly, growing experimental evidences leading to hypothesize the existence of a close anatomo-functional interaction between NT and Dopaminergic system at basal ganglia level (Nemeroff CB., 1985), with a key role for the neurotensin receptor type 1. NTS1 is widely expressed on dopaminergic neurons of substantia nigra, in the striatum on dopaminergic striatonigral terminals and on glutamatergic terminals from cortical inputs. Because of the involvement of an over-activation of DA system in the development of neurological disorders such as schizophrenia, psychosis and dyskinesia, a strong attention was given to the study of complex interactions between NTS1 and D_2 dopamine receptor. In more recent studies, a possible mechanism of interaction between the two systems has reached a general acceptance that is an NTS1/D_2 antagonistic receptor-receptor interaction (Fuxe K., 1995; Tanganelli S., 1994; Ferraro L., 2001; Antonelli T., 2007). In view of the essential involvement of DA system at basal ganglia level in the eziopathogenesis of psychiatric disorders as schizophrenia, Nemeroff & coll. has suggested a potential antipsychotic role for NTS1 agonists (Caceda R., 2006; Ferraro L., 2007).

Only recently, experimental data obtained in our laboratory has shown that NT plays a crucial role also in regulating glutamatergic transmission. Such experimental evidences, obtained both in in vivo (Ferraro L., 1998, 2000) and in vitro models (Ferraro L., 2000; Antonelli T., 2004) allow a neurochemical,

biochemical and morphological characterization of NT modulatory activity on glutamatergic system. In fact, NT activation of NTS1 receptor seems to be able to amplify NMDA receptor signalling, even at threshold concentrations (Antonelli T., 2004)

Glutamate toxicity essentially contributes to neuronal degeneration in many acute CNS diseases, including ischemia, trauma, and epilepsy, and may play a role in chronic neurodegenerative diseases, such as PD and many others. In line with this view, the aims of the present work were:

I) to point out the potential involvement of NT in the pathological events induced by an excess in glutamate extracellular levels. So, an in vitro model of cerebral ischemia was obtained by the exposure of primary cortical cell cultures to oxygen and glucose deprivation (OGD), a well characterized model of acute neurodegeneration induced by an excitotoxic insult (Strasser U., 1995). By using several morphological and biochemical techniques we want to higlight a relationship between glutamate extracellular levels, neuronal death mechanisms and NTS1 signalling activation.

II) to confirm the hypothesized mechanism of receptors interaction between NTS1 and NMDA receptors, using both in vitro and in vivo experimental models. Such experiments were performed at cortical and striatal levels because of the involvement of these cerebral areas in physiological and pathological conditions relatively to locomotor control pathways. Finally, we have performed a preliminary set of studies using an in vivo model of Parkinson's disease (6-OHDA-lesioned rats) to investigate if the NTS1 specific antagonist, SR48692 could have neuroprotective effects and if there is a relevance of NTS1/NMDA receptors interaction in the striatum of 6-OHDA-lesioned rats.

EXPERIMENTAL WORK

3. MATERIALS AND METHODS

Animal Care

All experiments were performed in accordance with the guidelines issued by the Italian Ministry of Health (Decreto Legislativo 116/92 and Decreto Legislativo 111/94-B), the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Male adult Sprague-Dawley rats were housed in cages in groups of five animals at a constant room temperature (20 °C) and exposed to a 12:12 h light–dark cycle (lights on at 06.00 a.m.). Food and water were provided ad libitum. Following delivery, the animals were allowed to adapt to the environment for at least 1 week before the experiment started

Primary Cultures of Rat Cortical Neurons

Primary cultures of cortical neurons were prepared from embryonic day 18 Sprague-Dawley rats. Removed cortices were dissected free of meninges and dissociated in 0.025% (w/v) trypsin. The tissue fragments were dissociated mechanically by repeated gentle pipetting through wide- and narrow-bore fire-polished Pasteur pipettes in culture medium (Neurobasal medium with supplements of 0.1 mM glutamine 10 lg/ml gentamicina, and 2% B27). The cells were counted and then plated on poly-L-lysine (5 lg/ml)-coated dishes at a density of 2.5 3 106 cells per dish and on poly-L-lysine (5 lg/ml)--coated multiwells (24 wells) at a density of 200 000 cells per well. In the dishes used for Hoechst 33258 nuclear staining, annexin V staining, and

microtubuleassociated protein 2 (MAP2) immunocytochemistry (see below), the cells were plated on poly-L-lysine (50 lg/ml)-coated glass coverslips. Cultures were grown at 37 °C in a humidified atmosphere of 5% CO2/95% air. Cytosine arabinoside (1 lM) was added at 5 days in vitro (DIV) to prevent glial cell proliferation. The cultures were maintained for 8 DIV before experiments.

OGD Exposure

The cultures were exposed to a transient OGD. To this purpose, in the OGD group, the culture medium was replaced with a glucose-free Krebs--Ringer bicarbonate buffer that had previously been saturated with 95% N2/5% CO2 and heated to 37 °C. The cultures were then put into an anaerobic incubator (pO2 < 2 mm Hg) with an atmosphere of 95% N2 and 5% CO2 and 98% humidity at 37 _C for 60 min. OGD was terminated by removing the cultures from the anaerobic incubator, by replacing the exposure buffer with oxygenated Krebs--Ringer bicarbonate buffer containing glucose and returning the cultures to the incubator under normoxic conditions. In control group, not submitted to OGD, the cultures were exposed to oxygenated Krebs-Ringer bicarbonate buffer containing glucose and placed in a humidified atmosphere of 5% CO2/95% air for 60 min. All the experiments were performed 24 h later.

Determination of Endogenous Extracellular Glutamate Levels

On the day of the experiment, the cells were rinsed twice by replacing the culture medium with Krebs-Ringer bicarbonate buffer (37°C). Thereafter, 3 consecutive fractions were collected renewing this solution (400 μL). The first 2 samples, collected every 50 min (before OGD), have been used to assess basal endogenous glutamate levels, whereas the third fraction was collected

24 h later. When required, NT and the NT receptor antagonist SR48692 were added to the cultures using the following experimental protocol: NT was added 50 min prior to OGD and maintained in contact with cells during the OGD. The NT receptor antagonist SR48692 was added 20 min prior to NT and maintained in contact with cells during NT and OGD exposure and during the 24-h period after OGD. NT and SR48692 alone were also tested. Endogenous glutamate levels have been quantified using a highperformance liquid chromatography/fluorimetric detection system, including a precolumn derivatization o-phthaldialdehyde reagent anda Chromsep 5 (C18) column. The mobile phase consisted of 0.1 M sodium acetate, 10% methanol, and 2.5% tetrahydrofuran, pH 6.5. The limit of detection for glutamate was 30 fmol per sample. The effects of the treatments on endogenous extracellular glutamate levels during the third fraction were reported and expressed as percentage changes of basal values, as calculated by the means of the 2 fractions collected prior to treatment.

Lactate Dehydrogenase Levels

The neuronal death was quantitatively evaluated measuring the lactate dehydrogenase (LDH) levels in the extracellular fluid 24 h after OGD exposure using the Cytotoxicity Detection Kit LDH (Roche, Basel, Switzerland). It has been previously established that LDH release correlates linearly with the number of damaged neurons after toxic insult. Sensitivity was 0.2--2 3 104 cells per well. Background LDH levels were determined in control cell cultures not exposed to OGD and were subtracted from all experimental values. The LDH level corresponding to complete neuronal death was determined by assaying sister cultures exposed to 1 mM glutamate for 24 h. The LDH values were expressed as percentage of the value found with complete neuronal death (100%). NT and SR48692 effects

on OGD-induced increase in LDH levels were evaluated by following the same procedure as described for endogenous glutamate experiments.

MTT Assay

The integrity of mitochondrial enzymes in viable neurons was determined with a colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) levels. In live cells, mitochondrial enzymes have the capacity to transform MTT into insoluble formazon. Sensitivity was about 2.5 3 104 cells per well. Twenty-four hours after OGD, cultures were incubated with MTT 5 mg/ml for 4 h at 37°C. The formazon was dissolved in isopropanol with 1M HCl and colorimetrically (absorbance at 570 nm) quantified. Neuronal viability corresponded to the value of the optical density read at 570 nm. The results were expressed as the percentage of neuronal viability measured in control cell cultures (100%). NT and SR48692 effects on OGD-induced change in mitochondrial enzymes activity were evaluated by following the same procedure as described for endogenous glutamate experiments.

Nuclear Staining with Hoechst 33258

Twenty-four hours after OGD exposure, cells were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS), and then incubated for 20 min at room temperature with Hoechst 33258 (1lg/ml in PBS). After rinsing with PBS, coverslips were mounted on slides with a solution containing 50% glycerol in 0.044 M citrate, 0.111 M phosphate buffer, pH 5.5, and visualized under a fluorescence microscope. The percentage of cells showing chromatin condensation (fragmented nuclei) was quantified by counting >3000 cells under each experimental condition (5 randomly selected fields per well, 9-18 wells per condition per experiment,

and 5 independent experiments). NT and SR48692 effects on OGD-induced increase of apoptotic nuclei were evaluated by following the same procedure as described for endogenous glutamate experiments.

Annexin V Staining

Annexin V staining was carried out using human annexin V-fluorescein isothiocyanate (FITC) kit (Bender MedSystems, Burlingame, CA) according to the manufacturer's instructions. Annexin V is a phospholipidbinding protein with high affinity for phosphatidyl serine. Annexin V staining was used to label phosphatidyl serine translocated to the outer membrane surface of several cell types undergoing apoptosis. Cells cultured on coverslips were washed twice with a buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2 and then incubated with a solution of 5 μL annexin V-FITC and propidium iodide (1 μg/mL). Stained cells were examined at the fluorescence microscope. To quantify the number of apoptotic cells, the annexin-positive/propidium iodide-negative [AN(+)/PI(-)] immunoreactive cells were counted, and the data were expressed as percent of counted cells. NT and SR48692 effects on OGDinduced increase of annexin V staining were evaluated by following the same procedure as described for endogenous glutamate experiments.

Caspase-3 Activity

Caspase-3 activity was measured in lysates of cortical neurons using the caspACE colorimetric assay system (Promega, Madison, WI) following the instructions of manufacturer. The colorimetric substrate N-acetyl-Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (Ac-DEVD-pNA) provided in the caspACE assay system is labeled with the chromophore p-nitroaniline that is released

from the substrate upon cleavage by caspase-3. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405 nm. Briefly, neurons were lysed in ice-cold lysis buffer for 20 min. After removal of cellular debris by centrifugation (10 min at 9000 rpm at 4°C), the supernatants were used to detect caspase-3 proteolytic activity. Samples were incubated with 200 μM caspase-3 substrate Ac-DEVD-pNA at 37°C for 4 h and then analyzed at 405 nm in a microtiter plate reader. The protein levels in the lysates were measured with BCA protein assay kit (Pierce Biotechnology, Rockford, IL). NT and SR48692 effects on OGD-induced changes in caspsase-3 activity were evaluated by following the same procedure as described for endogenous glutamate experiments.

MAP2 Immunoreactivity

The neuronal damage was also performed by neurons immunochemical numeration through MAP2 immunocytochemistry. On DIV 8, cells were rinsed in 0.1 M PBS and then fixed using 4% paraformaldehyde in Sorensen's buffer 0.1 M, pH 7.4, for 20 min. After rinses in PBS (3 times for 5 min each), the cells were incubated overnight at 4°C with the primary antibody rabbit anti-MAP2. Anti-MAP2 antibody was diluted 1:1000 in PBS containing 0.3% Triton X-100 (v/v). The cells were then washed 3 times with PBS and incubated with rhodamine-conjugated antirabbit antibody (Chemicon, Temecula, CA) diluted 1:100 in PBS containing 0.3% Triton X-100 for 60 min at room temperature. After 3 washes in PBS, the cells were mounted in glycerol and PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope. Investigation of MAP2 aggregations in dendrites was performed with the 3100 magnification objective and on 30 randomly chosen fields in each coverslip. Subsequently, the number of aggregations was counted and referred to 100 lm of dendrite

length (Image-Pro Plus 4.1; Immagini e Computer, Milan, Italy) (Pirondi S., 2005). NT and SR48692 effects on OGD-induced increase of MAP2 immunoreactivity were evaluated by following the same procedure as described for endogenous glutamate experiments.

In vivo microdialysis

On the day of surgery, the animals, kept under halothane anaesthesia (1.5% mixture of halothane and air), were mounted in a David Kopf stereotaxic frame with the upper incisor bar set at -2.5 mm below the interaural line. After exposing the skull and drilling an hole, a microdialysis probe of concentric design (CMA 12, MW cutoff 20.000 daltons, Carnegie Medicin, Sweden) was implanted into the right or the left cerebral cortex or dorsolateral striatum (outer diameter 0.5 mm; length of dialysing membrane 2 mm). The coordinates relative to the bregma were respectively for cerebral cortex and striatum: A:-1.8; L:±3.2; V:-3.0 and A:+0.8; L:±3.5; V:−5.5 (Paxinos and Watson, 1986).

Following the implantation, the probe was permanently secured to the skull and 36 h later the release experiment was performed. On the day of the experiment, the probe was perfused with Ringer solution (in mM: Na^+ 147; K^+ 4; Ca^{++} 1.4; Cl^- 156; Glucose 2.7) at a constant flow rate (2 µl/min) by using a microinfusion pump.

The collection of perfusate samples started 300 min after the onset of perfusion to achieve stable dialysis glutamate levels and perfusates were collected every 20 min. After three stable basal values were obtained, NMDA was locally perfused for 15 min while NT was perfused into the striatum for 60 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values

before treatment. Each point represents the mean ± SEM of 6–8 animals. Endogenous extracellular glutamate levels have been quantified using a highperformance liquid chromatography/fluorimetric detection system as described above.

6-OHDA Lesion

Animals were unilaterally lesioned by an injection of 6-OHDA (8 μg dissolved in 4 μl of a 0.02% ascorbic acid saline solution) into the right substantia nigra, sham operated animals were unilaterally injected in the same way with the same saline/ascorbic acid solution.

The rotational model (Ungerstedt U., 1970) was employed to select the rats which had been successfully lesioned. 2 weeks after the 6-OH-DA or saline injection, rats were primed with a 50 μg/Kg s.c. dose of apomorphine and subsequently treated twice a week with a test dose of apomorphine (25 μg/Kg s.c.). Only animals showing turning behaviors > 400 rotations/40 min were chosen for the experiments. This behavior has been correlated to a 95% depletion of dopamine in the striatum (Cadet JL., 1992). All groups of sham-operated animals failed to rotate in response to apomorphine injection.

Turning behaviour test

Animals were subdivided into three groups: untreated rats, rats treated with the NTS1 receptor antagonist SR48692 (0.1 mg/kg i.p.) or its solvent (0.01% DMSO–saline solution: vehicle) from one-week before until one-week after the lesion. The same protocol of treatments was followed in sham-operated rats. At 1, 2 and 3 weeks after the lesion, the turning behaviour of animals in response to apomorphine was tested.

Materials

Animals were purchased from Stefano Morini (Reggio Emilia, Italy), kept under regular lighting conditions (12 hr light/dark cycle), and given food and water *ad libitum*.

The culture dishes were purchased from Nunc A/S (Roskilde, Denmark). Neurobasal medium and B27 were obtained from Gibco (Grand Island, NY). Poly-L- lysine, trypsin, cytosine arabinoside, gentamycine sulfate, glutamine, L-glutamic acid, MTT, and Hoechst 33258 were obtained from Sigma Chemical Co., St Louis, Missouri. Anti-MAP2 antibody and rhodamine-conjugated antirabbit antibody were purchased from Chemicon. NT from Peninsula Laboratories Europe Ltd (Merseyside, UK) was dissolved in Krebs solution just before testing and used only once. SR48692 (2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl)carboxylamino]tri-cyclo (3.3.1.1.3.7)-decan-2-carboxylic acid) (Sanofi-Aventis, Montpellier, France) was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was < 0.001%; when required, the DMSO vehicle was added alone or together with NT, and no changes in glutamate efflux, LDH levels, mitochondrial enzymes activity, chromatin condensation, caspase-3 activity, annexin V staining, and MAP2 immunoreactivity were observed.

NT (Peninsula Laboratories, Belmont, CA) and was dissolved in Ringer's solution just before testing and used only once. 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl)carboxylamino]tricyclo)3.3.1.1.$^{3.7}$)-decan-2-carboxylic acid (SR48692; Sanofi Recherche, Montpellier, France) was dissolved in dimethylsulfoxide and intraperitoneally injected or diluted in the Ringer's solution (final concentration of dimethylsulfoxide was <0.001%). When required, the DMSO vehicle was perfused in either control animals or in combination with NT perfusion.

All other chemicals used were of analytical grade and purchased from Sigma or Merck (Darmstadt, Germany)

Statistical Analysis

Results are expressed throughout as means ± standard error of mean. The statistical analysis was carried out by ANOVA analysis of variance followed by the Newman-Keuls test for multiple comparisons. $P < 0.05$ was the accepted level of significance.

4. RESULTS

4.1 Results I

Since the data obtained to date suggest a strengthening NT-mediated on several functions exercised by glutamate in the central nervous system, we wanted to deepen his possible involvement in glutamate-induced neurodegenerative mechanisms. Experiments on cultured rat cortical neurons exposed to oxygen and glucose deprivation, an in vitro model of cerebral ischemia were conducted.

Endogenous Extracellular Glutamate Levels

As shown in Figure 15 , endogenous extracellular glutamate levels, measured 24 h after OGD, were significantly increased (437 ± 35% of the respective basal value, n = 15) in respect of control group (293 ± 21% n = 10). Succesive addition of NT (100 nM, ineffective on control cultures) to the cell cultures exposed to OGD was associated with a supplementary enhancement of the OGD-induced increase of endogenous extracellular glutamate levels (540 ± 47% n = 14). The simultaneous treatment with the NT antagonist SR48692 (100 nM, ineffective on control cultures) prevented the previous effect of the peptide with a reduction in glutamate efflux (270 ± 28% n = 14) to a level not significantly different from the control group. The use of SR48692 by itself significantly reduce the OGD-induced increase of endogenous extracellular glutamate levels (332 ± 37%, n = 14)

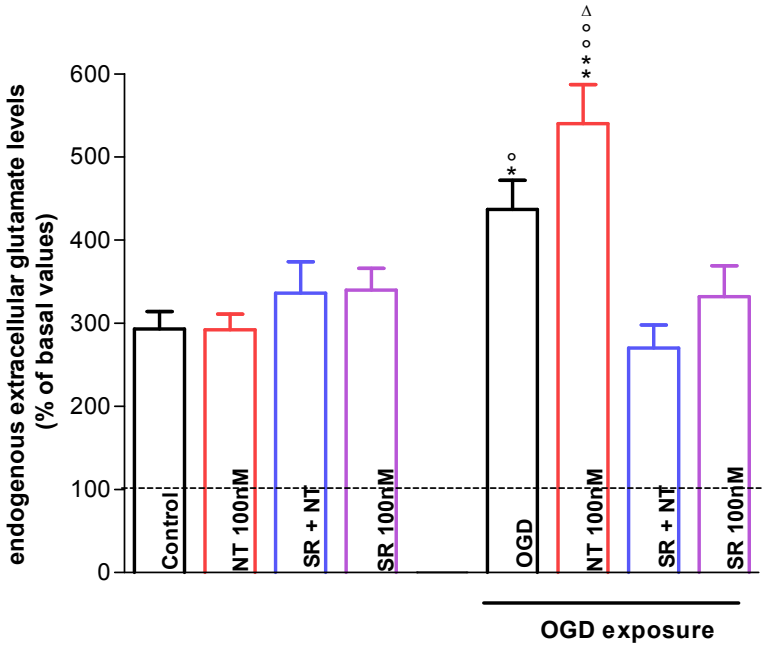


Fig. 15: Effects of NT, SR48692 (SR), and NT + SR on endogenous extracellular glutamate levels in cortical cell cultures not exposed and exposed to OGD. Two samples collected every 50 min, have been used to assess basal glutamate levels. A third fraction was collected 24 h later. The effects of the treatments on the endogenous extracellular glutamate levels in the third fraction are reported and expressed as percent changes of basal values, as calculated by the means of the 2 fractions collected prior to treatment. *P<0.05 and **P<0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °P<0.05 and °°P<0.01 significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}$ P<0.05 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Cell death: LDH Release

LDH release quantification was assesd in order to define if the OGD model induces neuronal death in our experimental system. The LDH evaluation is an indirect mesurment of the loss of cell membrane integrity, an essential step in LDH release mechanism and an acute cause of cell death (Figure 16).

The OGD experimental group show a significant increase of LDH release (66 ± 2.6% of complete neuronal death, n = 35) with respect to sister cell cultures not exposed to OGD (37% ± 2.8% n = 28). The addition of NT (100 nM, ineffective on control cultures) to the cultures medium further enhanced the OGD-induced increase of LDH release (82% ± 2.8% n = 23). The simultaneous treatment with the NT antagonist SR48692 (100 nM, ineffective on control cultures) prevented the previous effect of the peptide and of the ODG exposure reducing LDH release to a control-like level (respectively 47% ± 2.5% n = 15 and 49% ± 2.5% n = 28).

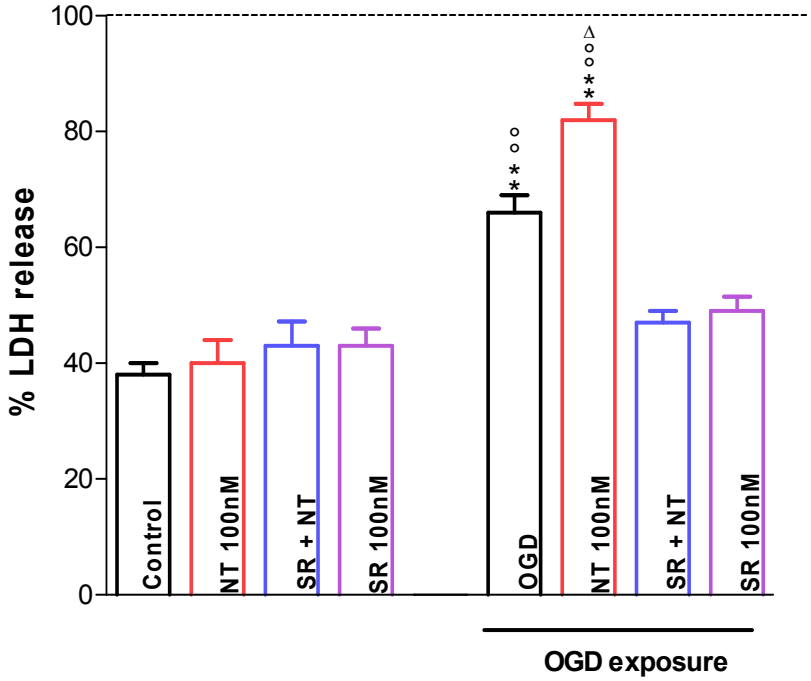


Fig. 16 : Effects of NT, SR48692 (SR), and NT + SR on LDH release in cortical cell cultures not exposed and exposed to OGD. The LDH values are expressed as percentage of the value found with complete neuronal death (100%), determined by assaying sister cultures exposed to 1 mM glutamate for 24 h. **P<0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °°P<0.01 significantly different from OGD + SR and OGD + SR + NT; ᐞ P<0.05 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Cell viability: MTT Assay

The MTT assay provides an indirect measurement of cell viability measuring the ability of mitochondria to convert MTT to formazon. This assay do not measures cell death per se, but an eventually relevant loss of mitochondrial function will be considered a as late indicator of apoptotic cell death (Figure 17). MTT absorbance measured in cultures exposed to OGD show an impairment of oxidative ability of mitochondria (MTT reduction) in respect of sister cultures not exposed to OGD (respectively 68% ± 4% of control value, n = 20; 0.125 ± 0.02, n = 20). NT addition (100 nM, ineffective on control cultures) to the cultures medium further impair the mitochondrial activity level (55% ± 2%, n =20). Both the OGD and OGD+NT effects result totally counteracted by the simultaneous addiction of SR48692 (100 nM, ineffective on control cultures) to the medium cultures (respectively 93% ± 4%, n = 18 and 91% ± 4%, n = 18).

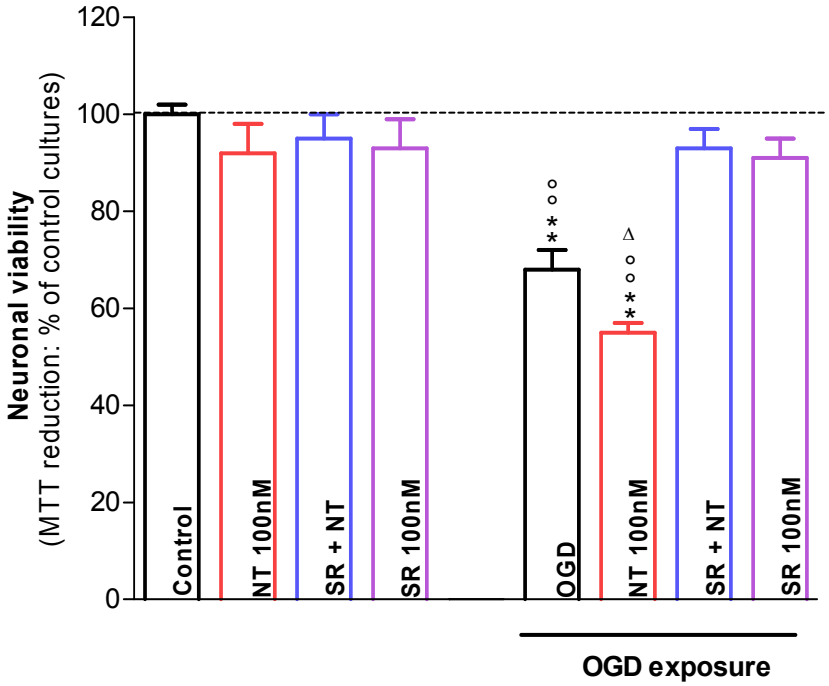


Fig. 17: Effects of NT, SR48692 (SR), and NT þ SR on neuronal viability (expressed as MTT reduction) in cortical cell cultures not exposed and exposed to OGD. **P < 0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °°P<0.01 significantly different from OGD + SR and OGD + SR + NT; ΔP<0.05 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Apoptotic cell death - I: Nuclear Staining with Hoechst 33258

Apoptosis is characterized by morphological and structural nuclear changes as chromatin condensation at the nuclear membrane, membrane blebbing without loss of integrity, and chromatin fragmentation. For this reason, the specific DNA stain, Hoechst 33258, was used to assess changes in chromatin and nuclear structure following OGD (Figure19).

Representative fluorescence photomicrograph (Figure 18A), nuclei of viable cells (control group, not exposed to OGD) exhibited a large and diffuse chromatin staining. In contrast, nuclei of cortical cells exposed to OGD showed a variety of abnormal morphologies including highly condensed and fragmented chromatin (Figure 18B). All nuclei with morphological abnormalities were considered ''condensed.''

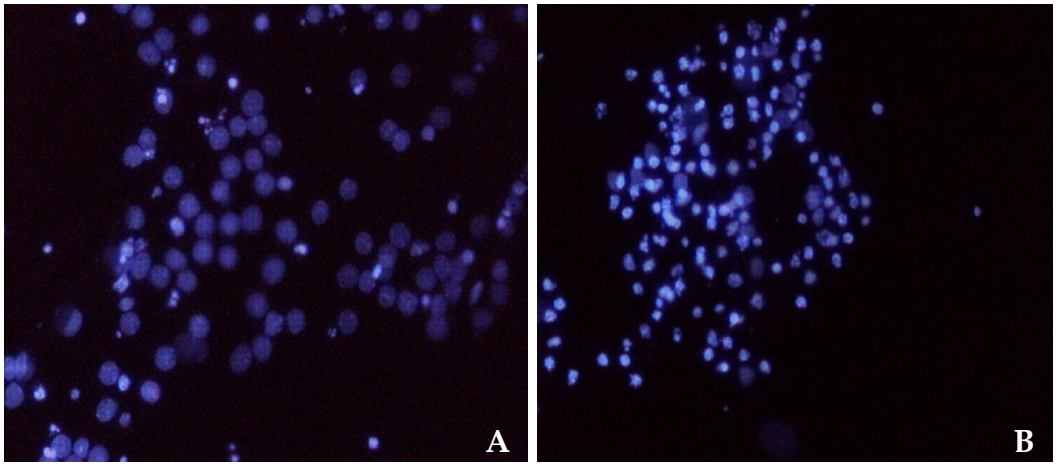


Fig. 18: A) Representative fluorescence photomicrographs of cells with nuclear fragmentation in cortical cell cultures not exposed to OGD. B) Cells exposed to OGD. The neurons were stained with Hoechst 33258 and observed in sampled fields under fluorescent microscope (magnification 20x).

In the OGD-exposed cultures, the percentage of cells with altered showing fragmented nuclei was significantly higher than that observed in control cell cultures not exposed to OGD (55% ± 3.8%, n = 18, and 32% ± 2.3%, n = 15, respectively). NT addition (100 nM, ineffective on control cultures) to the cultures medium further increase the OGD-induced amplification of fragmented nuclei (71% ± 3%, n = 9). The selective NT antagonist SR48692 (100 nM, ineffective on control cultures) the NT and/or OGD -induced nuclear fragmentation was counteracted (43% ± 1.5%, n = 8; 41 ± 1.4%, n = 9 respectively).

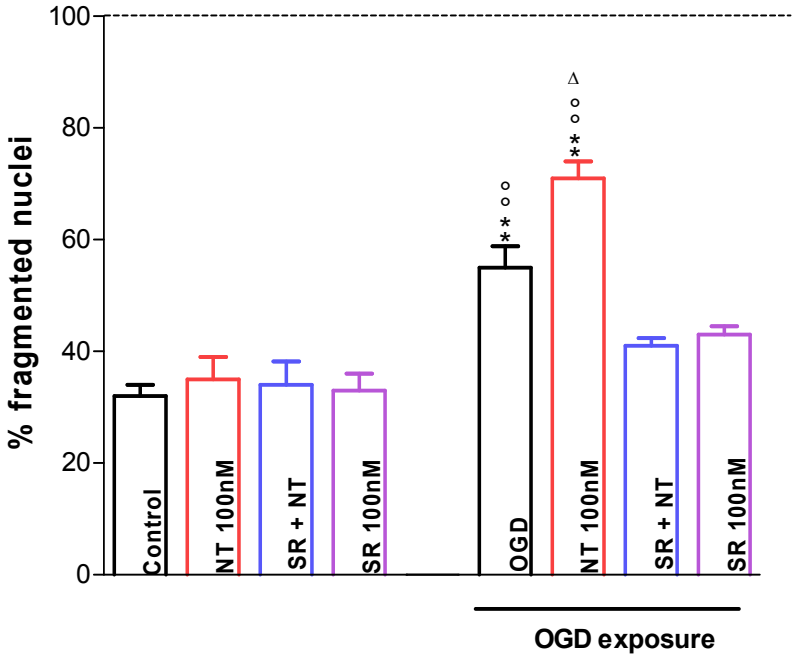


Fig. 19: Effects of NT, SR48692 (SR), and NT + SR on the percentage of fragmented nuclei in cortical cell cultures not exposed and exposed to OGD. **P<0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °°P<0.01 significantly different from OGD + SR and OGD + SR + NT; Δ P<0.05 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Apoptotic cell death - II: Annexin V Staining

In consideration of the above results, annexin V assay was used to confirm the presence of the apoptotic component in OGD-induced neuronal cell death. Annexin V assay is based on a specific apoptotic feature that is the translocation from the inner leaflet to the outer leaflet of the plasma membrane of phosphatidyl serine (PS) residues. The cells stained by annexin V but not by propidium iodide [AN(+)/PI(–)] are those that undergo apoptotic cell death (Figure 21).

A typical staining closely localized upon the plasma membrane highlight the presence of few cells annexin positive in control cultures not exposed to OGD (Figure 20A). On the other hand, sister cultures exposed to OGD shown a diffuse markedly increase in the annexin V staining (Figure 20B).

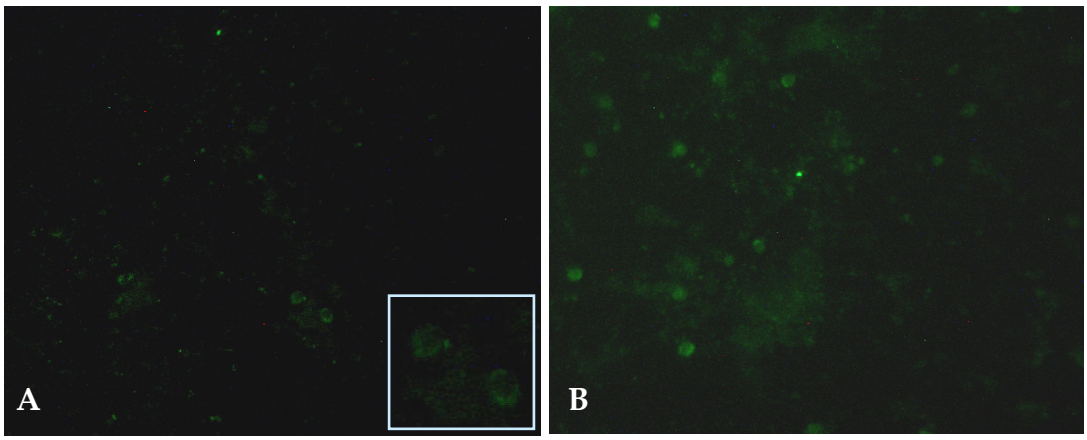


Fig. 20: A) Representative fluorescence photomicrographs of AN(+)/PI(–) immunoreactive cells in cortical cell cultures not exposed to OGD. A detail showing the characteristic immunostaining is reported with magnification 40x. B) Cells exposed to OGD. The neurons were stained with Annexin V and observed in sampled fields under fluorescent microscope (magnification 20x).

In the OGD-exposed cultures the number of AN(+)/PI(–) immunoreactive cells increased 9 ± 0.9% (n = 9) in respect of control sister culture (4.5%± 0.5%, n = 9). NT addition (100 nM, ineffective on control cultures) to the cultures medium further increase the OGD-induced amplification of annexin V staining (13% ± 0.8%, n = 9;). The selective NT antagonist SR48692 (100 nM, ineffective on control cultures) counteract the NT-induced nuclear fragmentation (9% ± 1%, n = 8) but not the OGD effect alone.

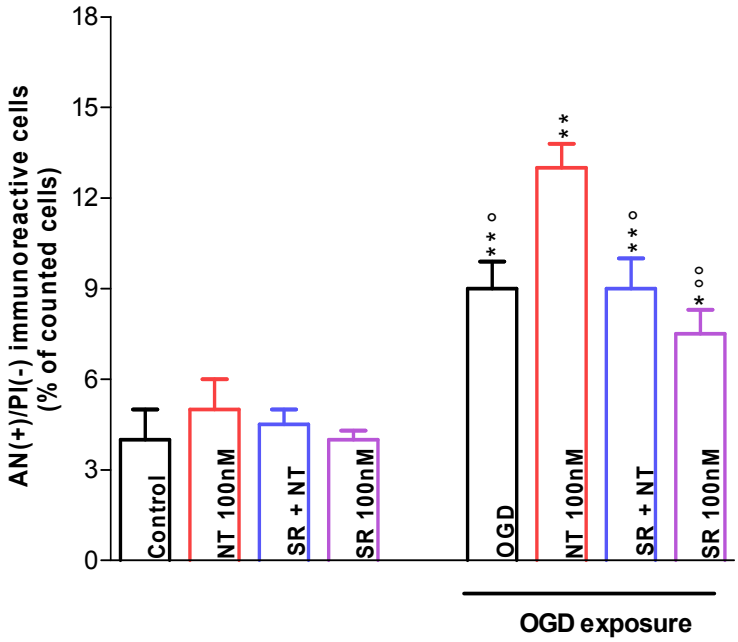


Fig. 21: Effects of NT, SR48692 (SR), and NT þ SR on the percentage of AN(+)/PI(-) immunoreactive cells in cortical cell cultures not exposed and exposed to OGD. *P<0.05 and **P<0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °P<0.01 significantly different from OGD + SR and OGD + SR + NT; °°P<0.01 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Apoptotic cell death - III: Caspase-3 Activity

Caspase-3 activity is an essential biochemical charateristic of both extrinsic and intrinsic apoptotic cell death. The activation of caspase-3, that is an executor caspase, indicate that a late phase of the process is in progress (Figure 22).

In cortical cell cultures not exposed to OGD, the caspase-3 activity was 6.9 ± 0.6 (pNA pmol/min/mg protein; n = 10). Exposure of cortical cell cultures to OGD induced an increase of caspase-3 activity (135% ± 10% of control value, n = 10). NT addition (100 nM, ineffective on control cultures) to the cultures medium further increased the OGD-induced amplification of caspase-3 activity (206% ± 18%, n = 10). The selective NT antagonist SR48692 (100 nM, ineffective on control cultures) counteracted the NT-induced caspase-3 activation (155% ± 16%, n = 10) but not the OGD effect alone.

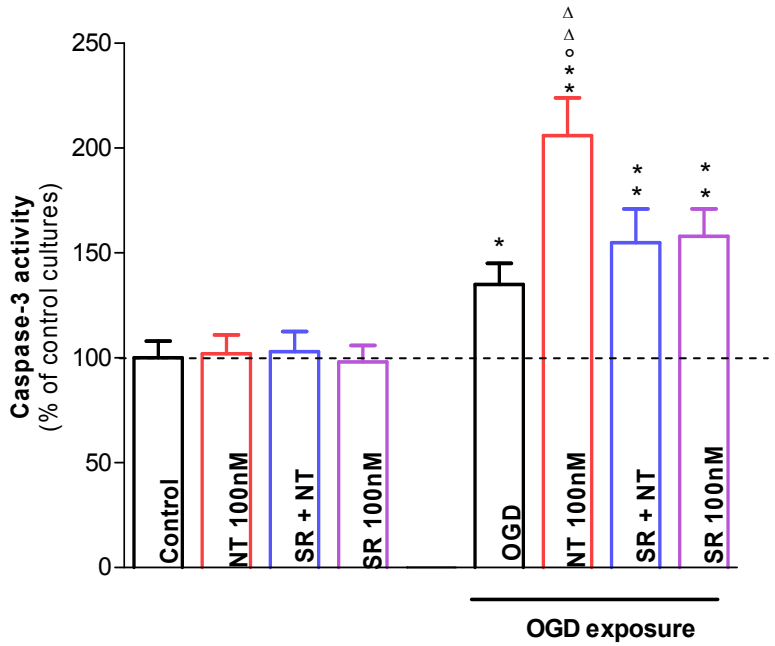


Fig. 22: Effects of NT, SR48692 (SR), and NT + SR on the caspase-3 activity expressed as percentage of control values in cortical cell cultures not exposed and exposed to OGD. *P < 0.05 and **P < 0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °P<0.05 significantly different from OGD + SR and OGD + SR + NT; $^{\Delta\Delta}$P<0.01 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Ischemia Neurodegeneration: MAP-2 Staining

MAP-2 family of proteins is an abundant group of cytoskeletal components predominantly expressed in neurons that have been proposed to play important roles in the outgrowth of neuronal processes, synaptic plasticity, and neuronal cell death. MAP-2 is depleted after in vivo and in vitro ischemia and for this reason, MAP-2 immunoreactivity may be considered an early indicator of ischemia-induced neurodegeneration.

In physiological conditions, the cultures showed a high number of healthy neurons and a neuronal network formed by highly arborized dendritic trees and an homogeneously diffused MAP-2 immunoreactivity of the cell bodies and dendrites (Fig. 23A, A'). A loss of MAP-2 immunoreactivity was observed after OGD particularly dendritic level, which often appeared to be truncated, as showed by the different distribution of MAP-2 immunoreactivity along the dendrites (Fig. 23D,D'). The number of MAP2 aggregations in dendrites was counted and referred to 100 μm of dendrite length sections (Figure 24).

In control cultures MAP-2 aggregations per length unit were 1.24 ± 0.98 aggregation per 100 μm (n = 10), whereas cultures exposed to OGD showed a marked increase (12.30 ± 1.10, n = 10). NT addition (100 nM, ineffective on control cultures) to the cultures medium further increased the OGD-induced increase in MAP-2 dendritic aggregations (17.55 ± 1.21, n = 11). The selective NT antagonist SR48692 (100 nM, ineffective on control cultures) counteracted the NT and/or OGD induced effects on dendritic structure and arborization (respectively 1.87 ± 0.69, n = 11; 2.12 ± 0.95, n = 11).

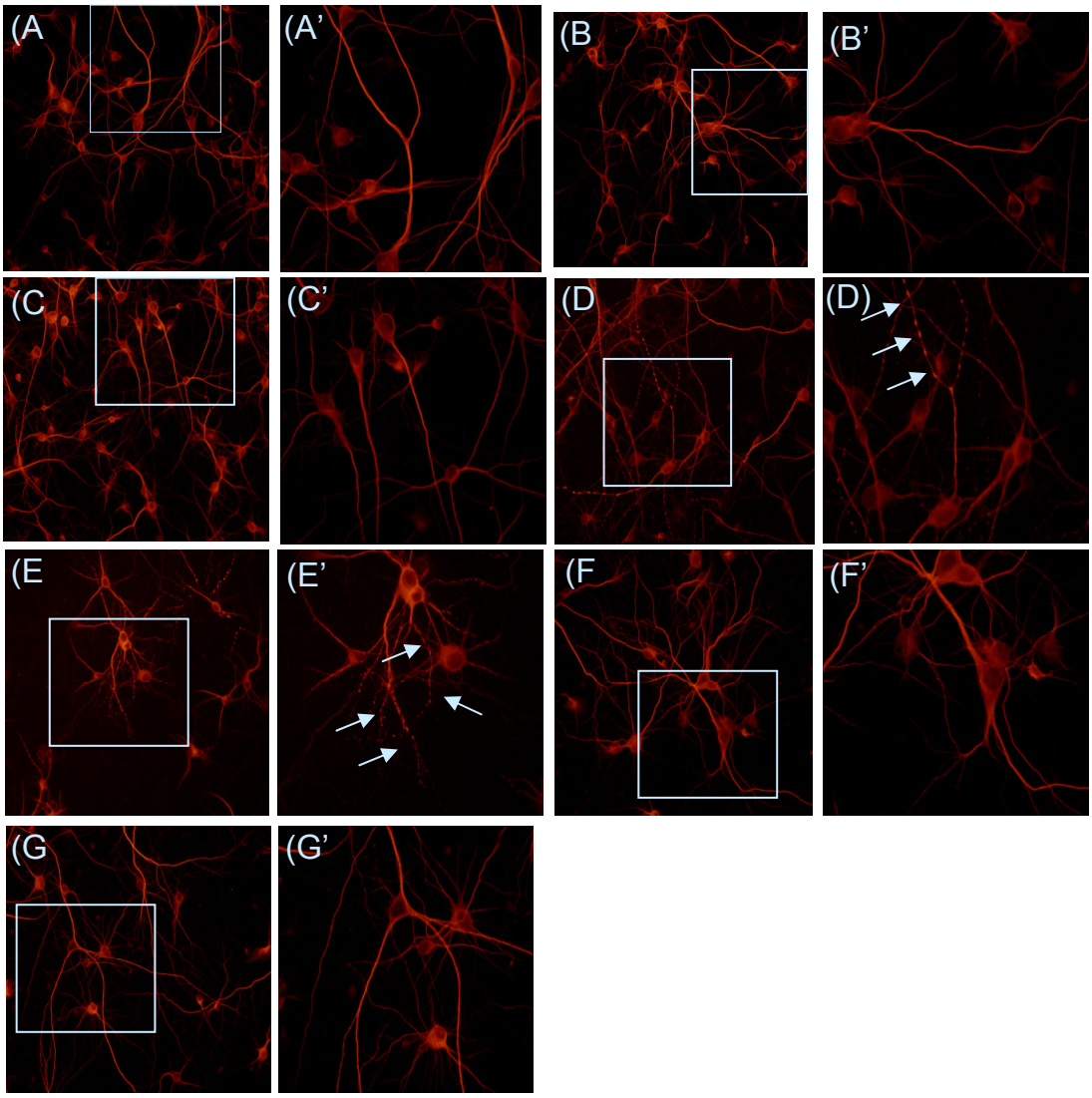


Fig. 23: Representative fluorescence photomicrographs of MAP2 immunoreactivity in cortical cell cultures not exposed (control: A, A′) and exposed to OGD (D, D′). Effects of NT 100 nM (B, B′) and SR48692 100 nM (C, C′) in cortical cell cultures not exposed to OGD. Effects of NT 100 nM (E, E′), SR48692 100 nM (F, F′), and NT 100 nM + SR48692 100 nM (G, G′) in cortical cell cultures exposed to OGD. Surviving neurons were stained with anti-MAP2 antibody and observed in sampled fields under fluorescent microscope 24 h after OGD (magnification 20x: A, B, C, D, E, F, G; magnification 40x: A′, B′, C′, D′, E′, F′, G′).

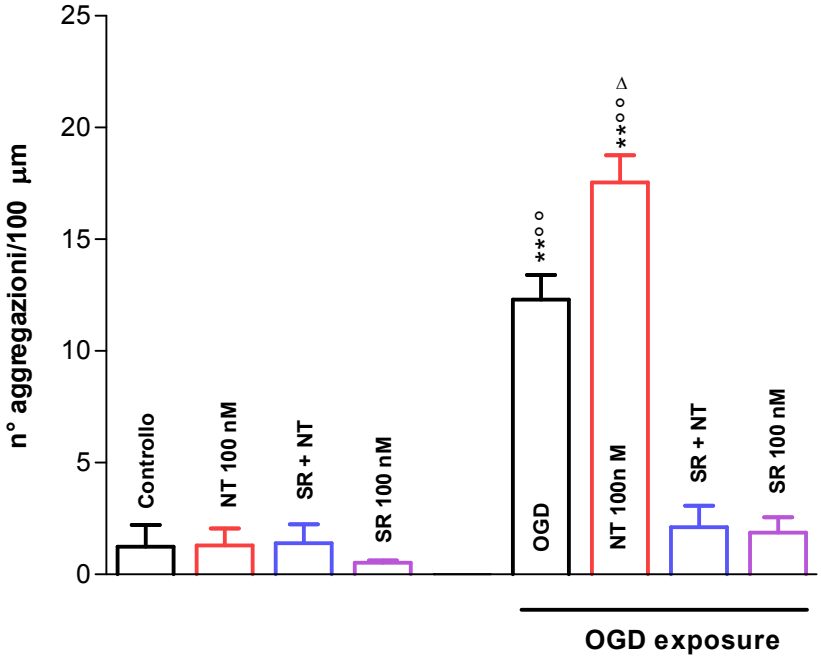


Fig. 24: Effects of NT, SR48692 (SR), and NT + SR on the number of MAP-2 aggregations per 100 µm length in cortical cell cultures not exposed and exposed to OGD. **P<0.01 significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); °°P<0.01 significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}$P < 0.05 significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Tiziana Antonelli, Maria Cristina Tomasini, Jacqueline Fournier, Roberta Mazza, Sergio Tanganelli, Stefania Pirondi, Kjell Fuxe and Luca Ferraro "Neurotensin receptor involvement in the rise of extracellular glutamate levels and apoptotic nerve cell death in primary cortical cultures after oxygen and glucose deprivation". **Cerebral Cortex 2008 18(8):1748-1757**

4.2 Results II

Evidence for the existence of NT/NMDA receptor interactions on the modulation of striatal and cortical glutamate transmission

Previous in vitro experiments of our group, performed on primary cell cultures of rat cortical neurons (Antonelli T., 2004), demonstrated that when ineffective concentrations of both NT (0.1 nM) and NMDA (0.01 μM), were simultaneously added into the cell cultures Mg^{2+}-free medium they induced a significant increase of glutamate extracellular levels. Furthermore, NT (0.1 nM) significantly amplified the NMDA (0.1 μM) induced increase in glutamate levels. The amplification of the NMDA receptor signalling induced by the presence of a threshold concentration of NT, was mediated by the activation of the NTS1 receptor subtype, since the pre-treatment with the selective NTS1 receptor antagonist SR48692 counteracted the enhancement induced by the co-application of NT and NMDA. To strengthen the hypothesis for a possible existence of an interaction between NT and NMDA receptors, it remains to be established whether also the pre-treatment with the NMDA receptor antagonist MK-801 counteracts the enhancement of glutamate levels induced by the co-application of NT and NMDA (Figure 25).

In vitro NT/NMDA receptor interactions on extracellular glutamate levels from primary cultures of rat cortical neurons

In primary cell cultures of rat cerebral cortex NT (10nM, iself ineffective) induced an increase (148% ± 4%, n = 9) in cortical glutamate levels in respect of sister control cultures (0.107μM ± 0.006μM, n = 8). This effect was partially counteracted when MK-801 (1μM, itself ineffective) was added to the medium 10 min before the peptide (125% ± 3%, n = 8). The selective NTS1 receptor antagonist SR48692 (100nM, itself ineffective) fully counteracted the NT-induced increase in cortical glutamate levels (107% ± 5%, n = 7).

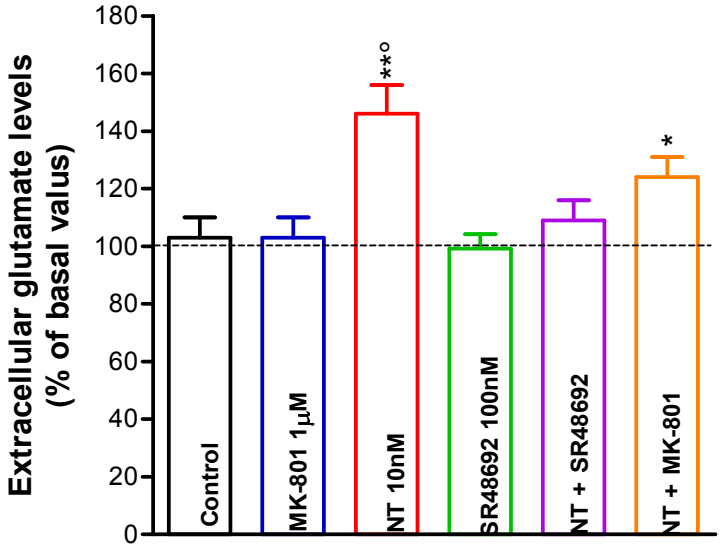
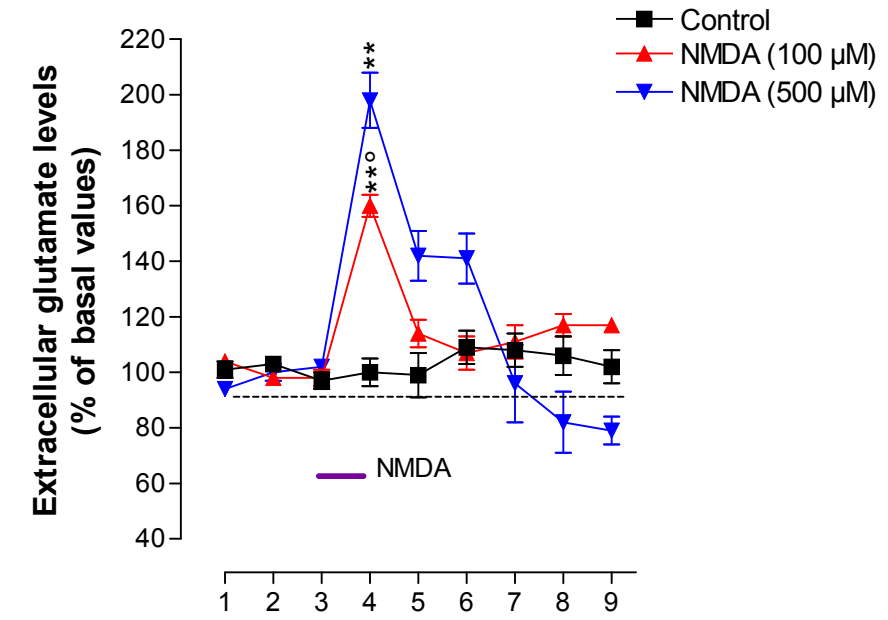


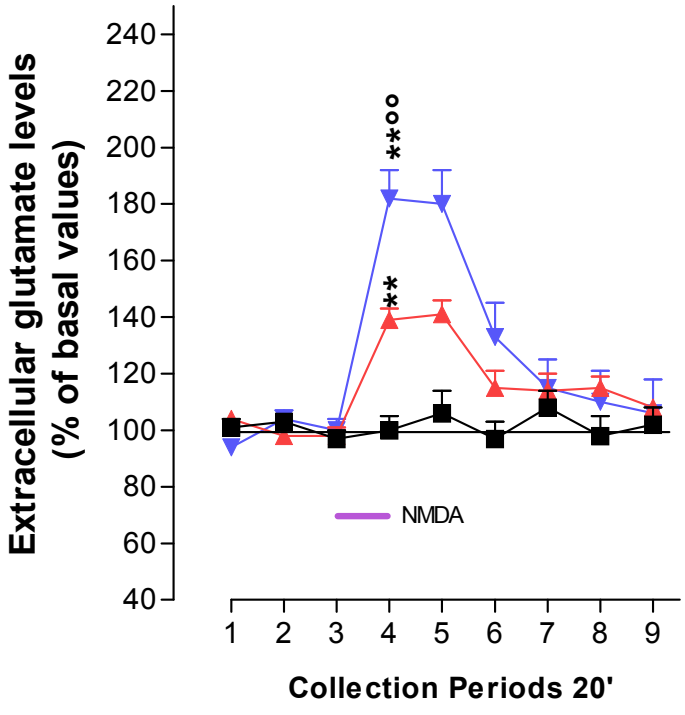
Fig. 25: Effects of NT (10 nM), alone and in combination with SR48692 (100 nM) or MK-801 (1 μM), on extracellular glutamate levels in primary cell cultures of rat cerebral cortex. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.107±0.006 μM), calculated from the mean of the first two fractions. NT was applied at the onset of the third fraction and maintained for 30 min, while MK-801 or SR48692 was added to the Mg^{2+}-free Krebs–Ringer solution 10 min or 20 min before the application of the peptide, respectively. Each column represents the mean±SEM of 10 or 12 experiments for each group. * P<0.05; **P<0.01 significantly different from control, MK-801, SR48692 and NT+SR48692; °P<0.05 significantly different from MK 801+NT according to ANOVA analysis of variance followed by the Newman-Keuls test for multiple comparisons.

In vivo NMDA receptor activity characterization studied on striatal and cortical extracellular glutamate levels of awake rats

A second set of experiments was conduced in order to characterize if also in vivo model, the effect of NMDA activity on extracellular glutamate levels (Figure 26).



A



B

Fig. 26: Effects of local perfusion on extracellular glutamate levels from the striatum (A) and cerebral cortex (B) of the awake rats. After three stable basal values were obtained, NMDA was locally perfused for 15 min as indicated by the respective solid bars at two different concentration (100 and 500μM). Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 6–8 animals. *p<0.05; **p<0.01; ***p<0.001 significantly different from control; °p < 0.05 and °°p < 0.01 significantly different from NMDA 500 μM; according to ANOVA analysis of variance followed by the Newman-Keuls test for multiple comparisons.

In vivo NT/NMDA receptor interactions studied on striatal and cortical extracellular glutamate levels of awake rats

A second set of experiments was conduced in order to verify if also in in vivo model, the previous NT modulatory effect on NMDA activity is still present.

In line with the above findings obtained in cortical cell cultures, in vivo microdialysis studies show that the local perfusion with NT (10 nM) enhances the glutamate releasing action of NMDA (100 μM) in the cortex and neostriatum of the awake rat (respectively 207% ± 5%, n= 8 ; 203% ± 4%, n = 9). The NT induced amplification of the NMDA receptor signalling is counteracted (respectively 140% ± 4%, n=8 and 135% ± 5%, n = 7) by the use of the NTS1 receptor antagonist SR48692 (100nM) (Figure 27 A, B).

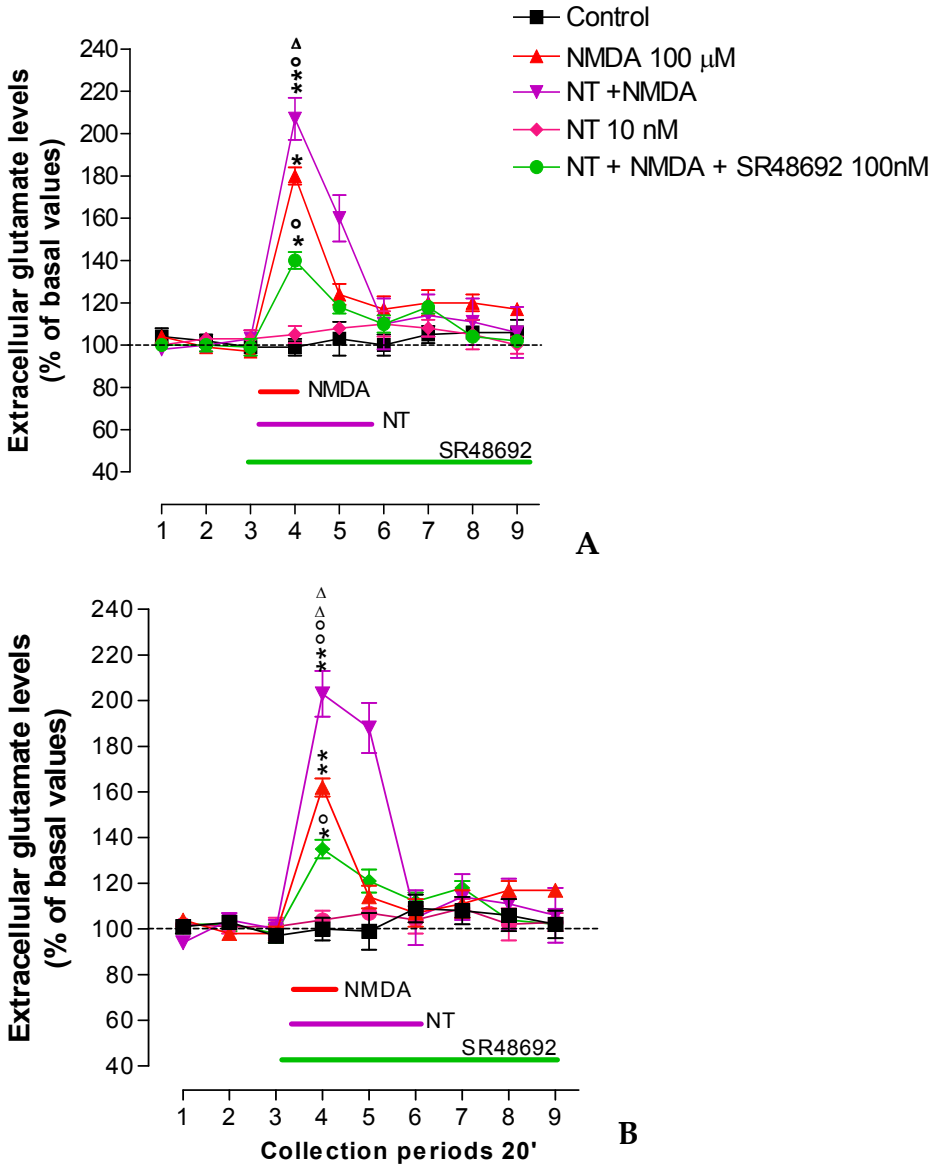
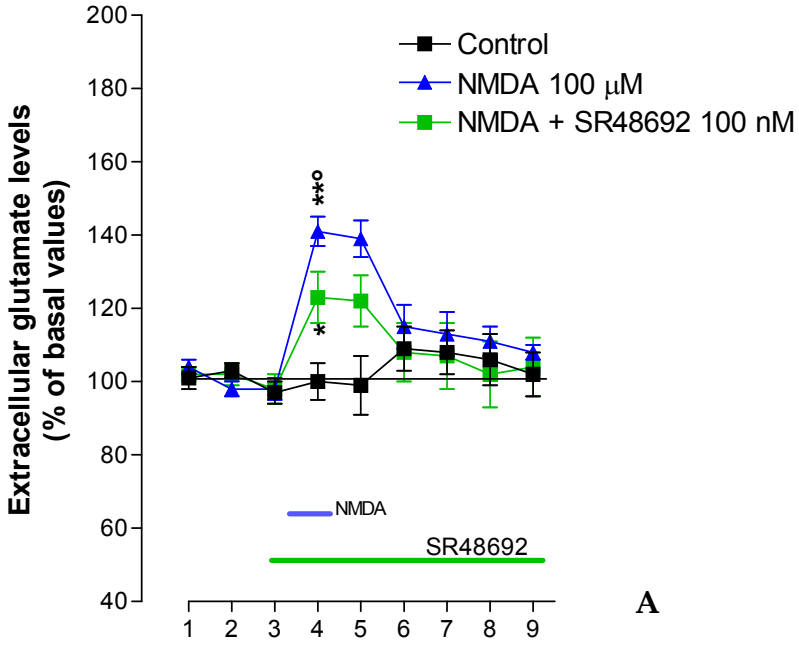


Fig. 27: Effects of local perfusion with NMDA in the presence of NT and NT + SR48682 on extracellular glutamate levels from the cortex (A) and striatum (B) of the awake rats. After three stable basal values were obtained, NMDA was locally perfused for 15 min while NT was perfused into the striatum for 60 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean± SEM of 6–8 animals. Panel A: *p<0.05; **p<0.01 significantly different from control as well as NT; °p<0.05; $^{\Delta}$p<0.01 significantly different from NMDA 100 µM; $^{\Delta\Delta}$P<0.01 significantly different from NT+NMDA+SR48692. Panel B: *p<0.05; **p<0.01 significantly different from control as well as NT; °p<0.05; °°p<0.01 significantly different from NMDA 100 µM; $^{\Delta\Delta}$P<0.01 significantly different from NT+NMDA+SR48692; according to ANOVA analysis of variance followed by the Newman-Keuls test for multiple comparisons.

SR48692 (100 nM, itself ineffective) alone reduces the NMDA (100 and 500 μM)-induced increase of extracellular glutamate levels in the cortex and striatum of awake rats (respectively Figure 28 A, B Figure 29 A, B).



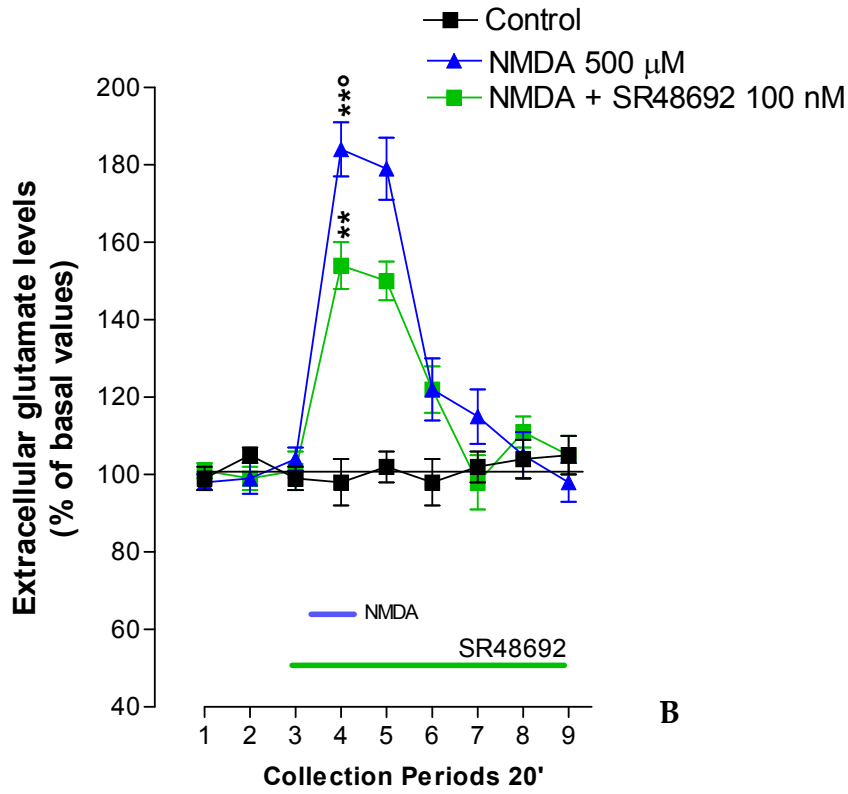


Fig. 28: Effects of local perfusion with NMDA 500 μM (A) and 100 μM (B) in the presence of the NTS1 receptor antagonist SR48692 on extracellular glutamate levels from the cortex of the awake rats. After three stable basal values were obtained NMDA was locally perfused for 15 min while SR48692 was perfused into the striatum for 120 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean±SEM of 4–8 animals. Panel A: *P<0.05; **P<0.01 significantly different from control; °P<0.05 significantly different from NMDA alone. Panel B: **P<0.01 significantly different from control; °°P<0.01 significantly different from NMDA + SR48692 group, according to ANOVA analysis of variance followed by theNewman-Keuls test for multiple comparisons.

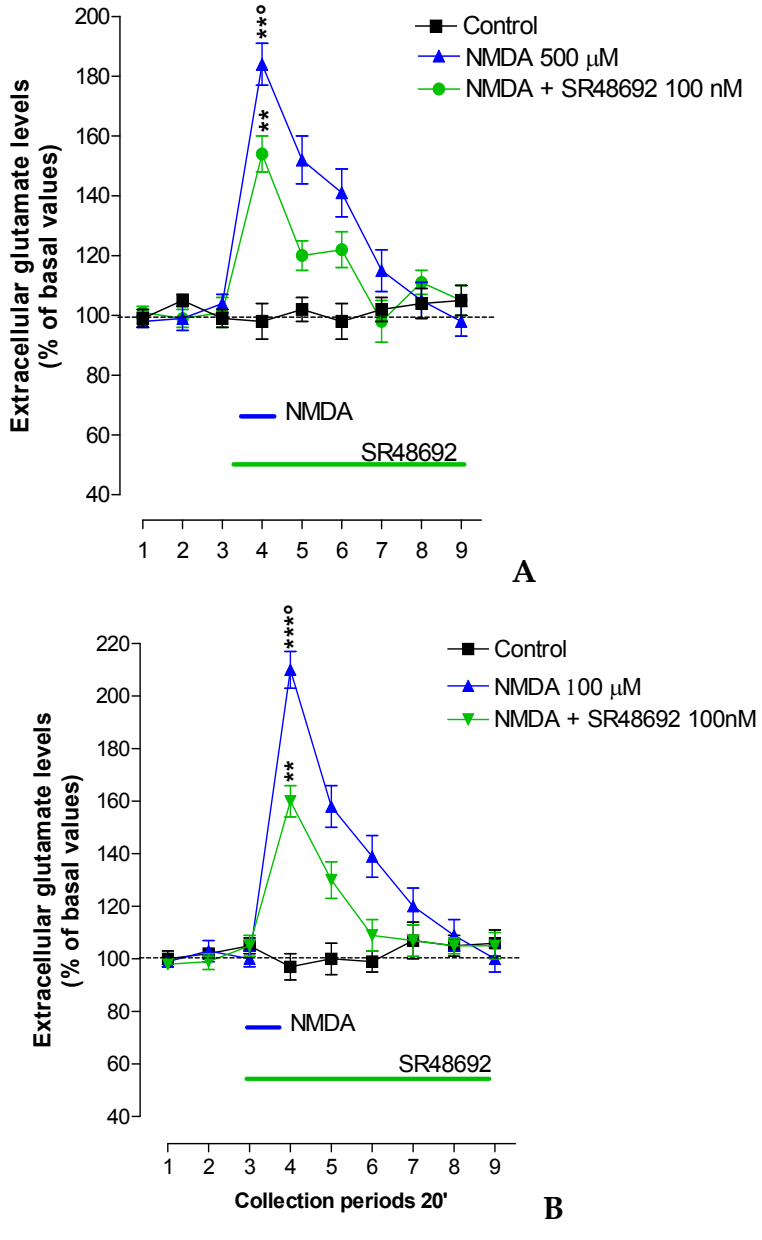


Fig. 29: Effects of local perfusion with NMDA 500 μM (A) and 100 μM (B) in the presence of the NTS1 receptor antagonist SR48692 on extracellular glutamate levels from the striatum of the awake rats. After three stable basal values were obtained NMDA was locally perfused for 15 min while SR48692 was perfused into the striatum for 120 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean±SEM of 4–8 animals. Panel A: **p<0.01 significantly different from control; °p<0.05 significantly different from NMDA+SR48692 group. Panel B: *p<0.05, **p<0.01 significantly different from control; °p<0.05 significantly different from NMDA+SR48692 group, according to ANOVA analysis of variance followed by theNewman-Keuls test for multiple comparisons.

In vivo NTS1/D_2 interaction studied on striatal extracellular glutamate levels of awake rats

In this new set of experiments a microdialysis probewas implanted into the striatum of awake rats (Figure 30). A pulse of 10' of high K+ (50 mM) Ringer solution significantly increased extracellular glutamate levels (basal values: 0.292± 0.033 M; K+ (50 mM) Ringer solution: 0.610±0.041; 209% ± 7%, n=7 of basal values). The perfusion with the dopamine D_2 agonist quinpirole (10 μM) significantly reduced the K+-evoked glutamate release (down to 163% ± 9%, n=7 of basal values). The addition into the perfusate medium of a low ineffective concentration (10 nM) of NT, in combination with the D_2 agonist (10 μM) counteracted the inhibitory effect of quinpirole on striatal K+-evoked glutamate outflow (216% ± 6%, n= 8 of basal values).

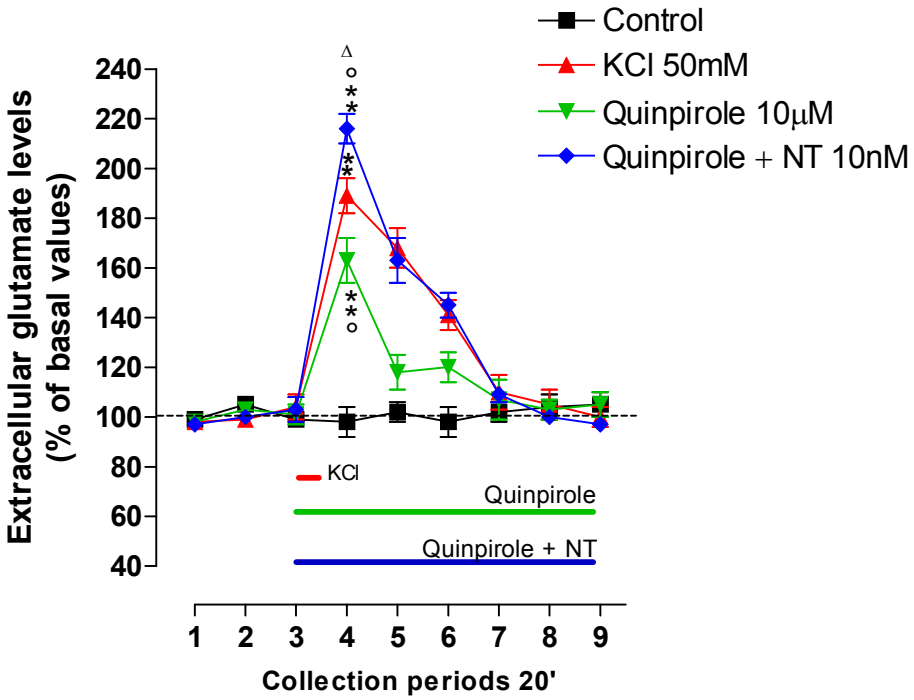


Fig. 30: Effects of local perfusion with KCl 50 mM, Quinpirole 10 µM and/or NT 10nM on extracellular glutamate levels from the striatum of the awake rats. After three stable basal values were obtained KCl was locally perfused for 10 min while the others treatments were perfused into the striatum for 120 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean±SEM of 4–8 animals. **p<0.01 significantly different from control; °p<0.05 significantly different from KCl group and $^{\Delta}$ p<0.05 significantly different from Quinpirole group, according to ANOVA analysis of variance followed by theNewman-Keuls test for multiple comparisons.

Relevance of the NT neuron systems in 6-OHDA-lesioned rats

In order to further investigate the possible relevance of NT in neurodegenerative process in vivo, a new set of experiments has been recently performed using an in vivo model of Parkinson's Disease.

Effects of chronic treatment with SR48692 on apomorphine-induced contralateral turning behaviour in hemiparkinson rats

In 6-OHDA-lesioned rats the apomorphine injection (0.1 mg/kg s.c.) produced a contralateral turning behaviour that significantly and progressively increased from week 1 to the 3rd week (respectively 70 ± 3, n= 7; 165 ± 4, n= 8; 195 ± 4, n= 8) following the lesion. In the SR48692-treated group, but not in the vehicle treated group, the apomorphine-induced rotational behaviour is significantly reduced at each time of evaluation (days 7, 14 and 21 post lesion) (Figure 31).

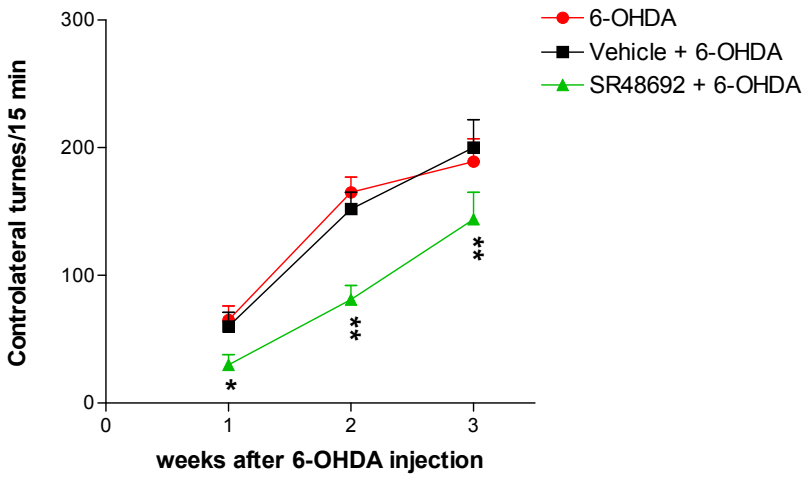


Fig. 31: Turning behaviour (total turns contralateral to the lesioned side per 15 min) induced by apomorphine (0.1 mg/kg, s.c.) in 6-OHDA-lesioned animals chronically treated with SR48692 (0.1 mg/kg) or its vehicle. Results are expressed as mean ± SEM. (n=14 -15 per treatment). *p<0.05; **p<0.01 significantly different from 6-OHDA as well as vehicle+6-OHDA groups according to repeated measures ANOVA analysis of variance followed by Newman-Keuls test for multiple comparisons.

Effects of chronic treatment with SR48692 on unilateral nigral 6-OHDA-induced modifications of striatal glutamate levels

A preliminary set of neurochemical experiments have been carried out in sham-operated animals and in rats chronically treated with SR48692 or its vehicle from one-week before until one-week after the 6-OHDA injection. In particular, the responsivity to a challenge with NMDA has been assessed (Figure 32).

In the groups of 6-OHDA-lesioned animals, control and vehicle-treated rats, intrastriatal perfusion (10 min) with NMDA (100 μM) induced a slight increase (122% ± 3% of basal values) in glutamate extracellular levels that was significantly lower than that observed in sham-operated animals (160% ± 6%). Interestingly, in 6-OHDA-lesioned rats chronically treated with SR48692, the effect of intrastriatal perfusion of NMDA (100 μM) induced an increase (141% ± 5%) in glutamate extracellular levels that was significantly higher with respect to that obtained in the group of 6-OHDA-lesioned rats but still lower to that observed in sham-operated rats. Any changes in the responsiveness to NMDA challenge were observed in all groups of sham-operated animals.

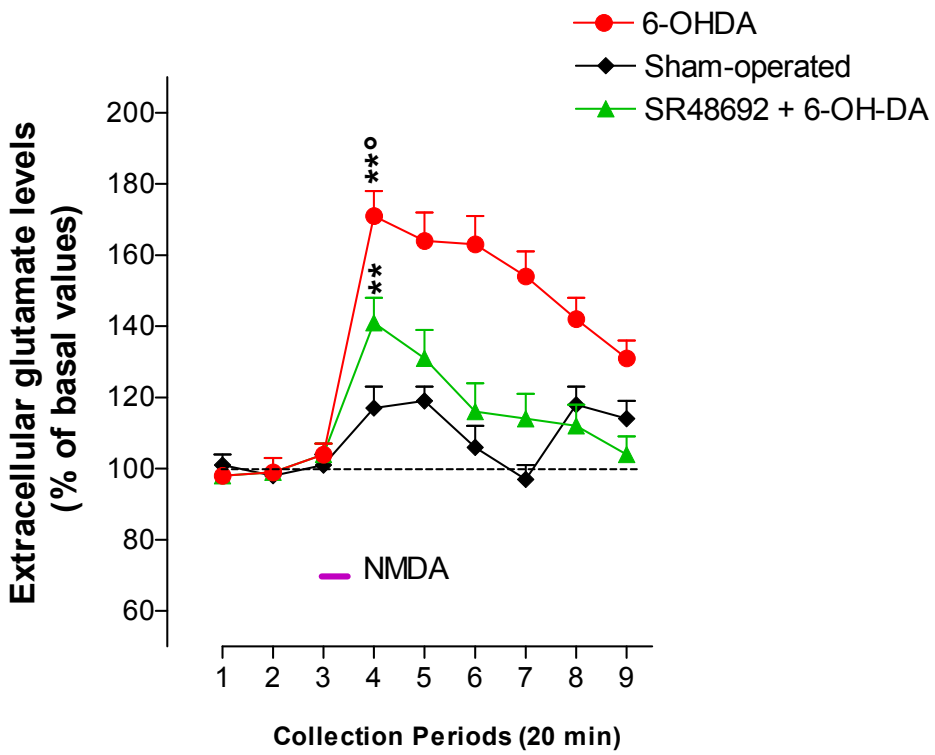


Fig. 32: Effects of local perfusion with NMDA 100 μM on extracellular glutamate levels from the striatum of 6-OHDA-lesioned animals chronically treated with SR48692 (0.1 mg/kg) or its vehicle. After three stable basal values were obtained NMDA was locally perfused for 15 min as indicated by the respective solid bar. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean±SEM of 4–8 animals. **p < 0.01 significantly different from control (saline); °p < 0.05 significantly different from SR48692 group according to ANOVA analysis of variance followed by theNewman–Keuls test for multiple comparisons.

Luca Ferraro, Maria Cristina Tomasini, Roberta Mazza, Kjell Fuxe, Jacqueline Fournier, Sergio Tanganelli, Tiziana Antonelli.
"Neurotensin receptors as modulators of glutamatergic transmission"
Brain Research Reviews 2008; 58(2):365-367

5. DISCUSSION

Wide ranges of experimental and clinical evidences have demonstrated that glutamate could play a key role in several neurodegenerative mechanisms, inducing neuronal death. The excessive stimulation of glutamatergic receptors, expecially NMDA receptors, is closely connected to neuronal acute and chronic cell death. Even if the exact mechanisms thereby the excitotoxic mechanism occur are not completely clarified, there is a general agreement concerning that phenomenon. Consequntly, an increasing number of experimental and clinical studies are intended to discover potentally new pharmaceutical targets able to prevent or at least reduce the neurotoxic effects of a glutamate extracellular excess.

In the last fifteen years, growing experimental evidences has revealed the existence of a functional interaction between NT, glutamate and dopamine in several brain areas. First of all, concearning the complex interactions that exist between NT and DA in the VTA, nucleus accumbens and cerebral cortex, in vivo experimental studies have highlighted a possible NTS1/D_2 receptor-receptor interaction (Fuxe K., 1992; Antonelli T., 2007a). On the other hand, in vivo microdyalisis esperiment has show that the NT locally perfused induce a dose-dependent increase of extracellular glutamate levels in the striatum of awake rats (Ferraro L., 2001). Coherently with further in vitro experiments obtained upon rat's cortical slices (Ferraro L., 2000) and cortical cell cultures (Antonelli T., 2004).

Moving from the large amount of experimental evidences obtained upon the neurotensin system physiological function, including its neuromodulatory role, for both dopaminergic and glutamatergic neurotransmission, the present work was intended to investigate if there is an involvement for NT system in several CNS pathological conditions. A specific attention was

given to such degenerative conditions that, at least in part, were induced by an excessive stimulation of NMDA glutamatergic receptors.

5.1 DISCUSSION I: PAPER II

It has been demonstrated in cultured cortical neurons that glutamate excitotoxicity via NMDA receptors induces apoptosis or necrosis depending on the intensity of the insult. Indeed, a mild glutamate insult leads to an apoptotic cell death, whereas an intense glutamate insult induces predominantly a necrotic process (Cheung NS., 1998). Therefore, endogenous compounds able to modulate glutamatergic transmission may interfere with glutamate-induced cell death. It has been also shown that the tridecapeptide neurotensin (NT) significantly enhances glutamatergic signalling in both in vitro (Ferraro L., 2000) and in vivo (Ferraro L., 1998, 2001) studies. These findings suggest a reinforcing action of NT on several functions exerted by glutamate in the central nervous system, in particular on the glutamate-mediated excitotoxicity (Antonelli T., 2007). Furthermore, an involvement of NT in modulating glutamate excitotoxicity has recently been demonstrated in primary cultures of mesencephalic dopamine (Antonelli T., 2002) and cortical (Antonelli T., 2004) neurons. In this context, an increase of NT levels in rat cerebral cortical areas has been demonstrated following focal ischemia by Allen & coll. (1995), suggesting a possible involvement of NT in ischemic brain damage.

Oxygen and glucose deprivation is an in vitro model of ischemia (Strasser U., 1995) used in the first part of this work to define the role of NT in OGD-induced neuronal death in cortical cell cultures and also to give a pathological correlate to the ability of NT to enhance NMDA-induced glutamate release (Antonelli T., 2004).

Using several different methods for measuring cell death in our experimental model, we were able to define causal relationship between the mechanisms that regulate programmed cell death and the death event itself. Biochemical methods such as LDH release and MTT enzyme activity, used to quantify the cell death, analyze opposite cellular events. Indeed, LDH release serves as an indicator of loss of cell membrane integrity and thus cell death. The MTT assay provides an indirect measurement of cell growth/viability through measurement of mitochondrial activity that is their ability of convert MTT to formazon. This assay allow measures of mitochondrial function and not cell death per se. The measurement of loss of mitochondrial function will be a late indicator of apoptotic cell death because mitochondria often remain intact until late in apoptosis. These biochemical methods present several drawbacks; indeed, both LDH release and MTT assay do not discriminate between apoptosis and necrosis. Thus, different methods are required to confirm apoptosis (Loo DT., 1998).

As says in introduction, apoptosis is characterized by specific cellular alterations (chromatin aggregation, membrane blebbing without loss of integrity, and chromatin fragmentation), enzymatical activity (caspases activation) and plasma membrane structure (PS residues translocation). For this reason, the specific DNA stain, Hoechst 33258, was used to assess changes in chromatin and nuclear structure following OGD. Furthermore, caspase-3 activity and Annexin V assay were used to confirm the presence of specific apoptotic component in OGD-induced neuronal cell death. Annexin V assay is based on the observation that during induction of apoptosis, phosphatidyl serine (PS) is translocated from the inner leaflet to the outer leaflet of the plasma membrane (Huerta S., 2007). Under the OGD condition, cultured cortical neurons exhibited Annexin V labeling on the periphery of their soma and dendrites while PI was excluded. These data confirm that PS

is externalized and so that the plasma membrane results intact, strongly supporting the occurrence of apoptosis. Thus, by measuring extracellular glutamate levels, LDH levels, mitochondrial dehydrogenase activity, apoptotic nerve cell death with Hoechst 33258, annexin V and caspase-3 activity, and MAP2 immunoreactivity, we provide biochemical and morphological evidence that NT, via the activation of NTR1, is involved in causing neuronal death upon OGD in cortical cultures.

The MAP2 family of proteins is an abundant group of cytoskeletal components that are predominantly expressed in neurons and have been proposed to play important roles in the outgrowth of neuronal processes, synaptic plasticity, and neuronal cell death. MAP2 is depleted after in vivo and in vitro ischemia (Li Y., 1995; Kuhn J., 2005), and for this reason, MAP2 immunoreactivity may be considered an early indicator of ischemia-induced neurodegeneration. (Buddle M., 2003).

In fact, the addition to the incubation medium of exogenous NT at nanomolar (ineffective) concentrations during OGD produces a further and significant enhancement of the LDH levels and a further significant decrease of mitochondrial activity with respect to the LDH and MTT levels observed during OGD alone. In line with the above finding, it has been shown that the NTR1 antagonist SR48692 can increase cell membrane integrity and cell viability.

Furthermore the peptide was able to enhance, whereas the NT receptor antagonist blocks, the OGD-induced increase in the number of fragmented nuclei and AN(+)/PI(–)immunoreactive cells as well as caspase-3 activity in the cortical cultures. In addition, the outgrowth of cortical dendrites, already altered after OGD (Matesic DF., 1994; Vanicky I., 1995), are further impaired in the presence of NT, as demonstrated by the MAP2 immunoreactivity analysis. The altered structure of neuronal dendrites, which may be observed

before neuronal death, is characterized by a reduction of outgrowth and branching of dendrites as well as by an increase of the number of MAP2 aggregations. This alteration in dendrite morphology is an early consequence of excessive glutamate release occurring during ischemia (Esquenazi S., 2002).

The involvement of NTS1 in the OGD-induced reduction of neuronal population and dendritic outgrowth is confirmed by the treatment with SR48692, which antagonizes the OGD-induced neurodegeneration. Thus, taken all together these biochemical and morphological results lead to hypothesize that NTS1 activation may contribute to nerve cell death induced by OGD with reduction of neuronal survival (Ioudina M., 2004), inducing apoptosis. Among the myriad of biochemical events triggered by cerebral ischemia, increase in Ca^{2+} influx, formation of free radicals, loss of adenosine triphosphate, etc., there is also an increase in extracellular concentration of neurotransmitters, especially of glutamate. In this context, it is well known that substantial elevation in extracellular glutamate levels and, consequently, the excessive stimulation of excitatory aminoacid receptors is implicated in the neuronal cell death occurring under these degenerative processes (Choi DW., 1988; Arundine M., 2004; Young C., 2004; Zipfel GJ., 2000).

Thus, in view of the results obtained in the present study, it could be suggested that one of the possible mechanisms that leads to NT-mediated apoptotic nerve cell death and NT-mediated reduction of dendritic outgrowth and branching could involve the ability of the peptide to modulate the glutamatergic transmission.

Such a hypothetical mechanism seems to be confirmed by the results indicating that during the OGD, the presence of NT in the medium induces a significant amplification in the rise of extracellular glutamate levels compared with that observed during OGD alone. This view is strengthened

by the result indicating that the NT receptor antagonist SR48692 counteracts the OGD-induced rise of the extracellular levels of glutamate reducing the endogenous levels of glutamate to the values observed in control neuronal cells. Interestingly, SR48692 is able to counteract some biochemical and morphological OGD-induced alterations also in the absence of exogenous NT. These findings can be explained by release of endogenous NT from neuronal and/or glial cells under OGD increasing NTR1 tone that can be blocked by the NTR1 receptor antagonist. In line with this explanation, a rise of NT immunoreactivity in certain brain regions has been found in the rat middle cerebral artery occlusion model of stroke (Allen GV., 1995). However, SR48692 by itself did not reduce the caspase-3 activation and the number of AN(+)/PI(–) immunoreactive cells induced by OGD. At the present, this discrepancy could be attributed to the different methodological approaches used to determine neurodegeneration but remain to be clarified.

It is known that protein kinase C, a transductional way activated by NTS1 receptor (Kinkead B., 2004), can increase the opening rate of the NMDA channels and recruit new NMDA channels to the surface membrane (Lan JY., 2001). This amplification of NMDA signaling by NT could explain the increase of MAP2 aggregations occurring after OGD. In fact, following NMDA receptor activation, the calcium influx, through the receptor ion channel complex, and the release of calcium from the mitochondria, through the activation of the 2Na+/Ca++ exchanger, trigger MAP2 degradation (Buddle M., 2003). In vitro experiment performed in mesencephalic cell cultures containing dopaminergic neurons that express functional NT receptors obtained by Antonelli & coll., demonstrate that the neurotoxic effects of glutamate are exacerbated by NT when the peptide is applied in combination with exogenous glutamate (Antonelli et al. 2002).

Moving from experimental evidences concearning the ability of NT to can enhance NMDA-induced excitotoxicity via activation of protein kinase C (Antonelli et al. 2004), it may be speculated that under the present pathological conditions, the peptide both by enhancing glutamate release and the NT-NMDA receptor interactions could amplify the glutamate signal transduction contributing to neuronal injury after both OGD and probably after ischemia. Thus, it is known that protein kinase C when activated can increase the opening rate of the NMDA channels and recruit new NMDA channels to the surface membrane (Lan JY., 2001). This amplification of NMDA signaling by NT could explain the increase of MAP2 aggregations occurring after OGD. In fact, following NMDA receptor activation, the calcium influx, through the receptor ion channel complex, and the release of calcium from the mitochondria, through the activation of the $2Na^{+}/Ca^{++}$ exchanger, trigger MAP2 degradation (Buddle M., 2003).

Such NT/Glutamate interaction was also highlighted by the means of an increase in ERK phosphorylation levels (Antonelli T. 2007). The autors reported that ineffective concentration of NT and NMDA, when co-administrated on cortical cultured neurons, are able to induce a significant increase in ERK^{P}/ERK^{T} levels, effect that result counteracted by SR48692

In conclusion, taken together the present results, obtained in the OGD model in cortical cultures representing an in vitro model of cortical ischemia, suggest that cortical NT receptor activation may contribute to neuronal injury after ischemia. The NT receptor antagonists could provide a new tool to explore the clinical possibilities and thus to move from chemical compound to effective drug.

5.2 DISCUSSION II: PAPER III

According to the hypothesis of a possible existence of an interaction between NT and NMDA receptors, the in vitro experiment performed on cortical cultured neurons with the NMDA receptor antagonist MK-801 have shown a significant inhibition of glutamate levels increase induced by the co-application of NT and NMDA. Furthermore, at higher concentration (10 nM) NT induced by itself an increase in cortical glutamate levels and this effect was only partially counteracted when MK-801 was added to the medium 10 min before the peptide. As expected, the selective NTS1 receptor antagonist SR48692 fully counteracted the NT-induced increase in cortical glutamate levels. The demonstration that the NT induced increase on cortical glutamate levels is in part antagonised by the blockade of NMDA receptors support the previous hypothesis of Antonelli & coll. (Antonelli T., 2004). The authors suggest that the enhancement of glutamate outflow induced by the peptide should 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. According to this possibility, Skeberdis & coll. (2001) demonstrated the existence of a mGluR1-mediated potentiation of NMDA receptors that involve the activation of PKC. Thus, considering all these in vitro findings it may be postulated that the NT-induced increase of glutamate transmission, associated with a PKC-induced amplification of NMDA receptor signalling, could represent one of the main mechanisms underlying the potential neurodegenerative action of NT.

Despite the well documented modulation of the DA transmission by NT (Nemeroff CB., 1985; Fuxe K., 1992; Rostene W., 1992; Legault M., 2002), there is a high degree of complexity in analyzing the role of NT in DA, glutamate and GABA release and the feedback loops between the different brain

regions involved in the extrapyramidal system. Recent in vivo microdialysis experiments provide evidence that NT directly enhance the neuronal activity of glutamatergic neurons in discrete rat brain regions of the basal ganglia such as the striatum, the substantia nigra and the medial prefrontal cortex (Sanz B., 1993; Ferraro L, 1998, 2001).

The observation that these effects are counteracted by the coadministration of the specific NTS1 antagonist SR48692 suggests that the NT-induced effects on glutamatergic transmission are mainly mediated by the activation of local NTS1 receptors. Our in vivo microdialysis studies demonstrated that local perfusion with a ineffective concentration of NT enhances the glutamate releasing action of NMDA both within the cortex and the neostriatum of the awake rat. The NT induced amplification of the NMDA receptor signalling is mediated via the activation of the NTS1 receptor subtype, since the NTS1 receptor antagonist SR48692 blocks the ability of the peptide to enhance the action of NMDA. What's more, the data presented show that the presence in the perfusate medium of SR48692 reduces the NMDA-induced increase of extracellular glutamate levels. Such an effect may be due to the fact that NMDA perfusion causes a rise in endogenous NT levels (Radke JM., 2001), which might contribute to the NMDA-induced ability to enhance glutamate transmission. Thus, from these in vivo findings it seems likely that SR48692 counteracts this “neurotensinergic” dependent part of the NMDA effect. In summary, these in vitro and in vivo findings show that NT increases glutamate release and simultaneously amplifies the glutamatergic receptor signalling, in particular the responsiveness of the NMDA receptors. These double activations are especially important for the toxic actions of glutamate and strengthen the hypothesis for a pathophysiological role of NT in glutamate-induced neurodegeneration.

Moving from the above observations the postulated neuroprotective effects of NTS1 antagonist SR48692 have been tested in a rat model of Parkinson's disease. Behavioural and neurochemical experiments have been carried out in different groups of animals: sham-operated rats and 6-OHDA unilaterally lesioned rats chronically treated with saline or with the NTS1 receptor antagonist. Animals unilaterally lesioned by an injection of 6-OHDA were tested at 1, 2 and 3 weeks after the lesion for the turning behaviour that is well correlated with the degree of DA dengeneration (Schwarting RK., 1996).

As expected, in 6-OHDA-lesioned rats the apomorphine injection produced a contralateral turning behaviour that significantly and progressively increased from week 1 to the 3rd week following the lesion. Interestingly, in the SR48692-treated group, but not in the vehicle-treated group, the apomorphine-induced rotational behaviour is significantly reduced at each time of evaluation. All groups of sham-operated animals failed to rotate in response to apomorphine injection. The findings of the present behavioural study indicate that systemic administration of NTS1 antagonist decreased the functional consequence of a partial dopaminergic lesion induced by intranigral application of the neurotoxin 6-OHDA in the rat.

Finally, in view of the above behavioural findings, a preliminary set of neurochemical experiments have been carried out in sham-operated animals and in rats chronically treated with SR48692 or its vehicle from one-week before until one-week after the 6-OHDA injection. In particular, the responsivity to a challenge with NMDA has been assessed.

In the groups of 6-OHDA-lesioned control and vehicle-treated rats, intrastriatal perfusion with NMDA induced a slight increase in glutamate extracellular levels, significantly lower than that observed in sham-operated animals. The glutamate extracellular levels increase observed in the 6-OHDA-lesioned rats chronically treated with SR48692, was significantly

higher with respect to that obtained in the group of 6-OHDA-lesioned rats but still lower to that observed in sham-operated rats.

In the above reported preliminary studies, behavioural and functional parameters have been evaluated in order to investigate the putative neuroprotective effects of SR48692 in an in vivo animal model of Parkinson's disease. The results obtained suggest that the NTS1 receptor antagonist partially protects dopaminergic neurons from 6-OHDA-induced degeneration.

Our first neurochemical observations are encouraging, demonstrating that a treatment with a specific NTS1 receptor antagonist, partially but significantly counteracts the degenerative process induced by 6-OHDA in the nigrostriatal pathway.

The observation that in rats chronically treated with SR48692 the excitatory response to a NMDA stimulus is partially restored on the striatal glutamatergic transmission may support a protective action of the NTS1 antagonist against 6-OHDA-induced DA neuron degeneration. The primary target for the neuroprotective action of SR48692 on DA nerve cells is likely to be its antagonistic action at NTS1 receptors leading to a partial protection or restoration of the dopaminergic nigrostriatal transmission as evidenced by the decrease in apomorphine-induced turning after intranigral injection of 6-OHDA. Indeed, as reported above, the activation of NTS1 by NT significantly enhances glutamatergic signalling in different brain areas including the substantia nigra and amplifies theNMDA receptor signalling (Antonelli T, 2004). This suggests a reinforcing action of NT not only on several functions exerted by glutamate in the central nervous system, but in particular on the glutamate-mediated excitotoxic mechanisms.

In conclusion, the present neurochemical, biochemical and behavioural data suggest that the use of selective NTS1 receptor antagonists in combination

with conventional drug treatments likely provides a possible novel therapeutic approach especially for the treatment of Parkinson's disease, underlined also by the high densities of NTS1 receptors found in the nigrostriatal DA cells.

5.3 FUTURE PERSPECTIVES

The well established NT neuromodulatory physiological role, for both glutamatergic and dopaminergic systems, has opened potentially new pictures in the study of eziopathogenesis of neurodegenerative disorders.

The NT involvement in dopaminergic mesencephalic and cortical neurons degeneration show by the data reported, seems due to an enhancement of the neurototoxic effect of glutamate, possibly via a rise of intracellular calcium levels and/or an amplification of NMDA receptors function by means of a receptor-receptor interaction mechanism.

We have provided in vivo evidence indicating that NT modulation of glutamatergic cortico-striatal neurons in the striatum takes place also via an antagonistic interaction with the dopamine D_2 receptor, as previously hypotesized (Fuxe K., 1992; Antonelli T., 2007a), and that NTS1 receptor antagonism partially protects from 6-OHDA-induced dopaminergic degeneration. So it could be speculated that there is a NTS1/NMDA/D_2 receptors interaction involved both in physiological and pathological CNS mechanisms. Such hypothesis require further studies to find out wich are the exact, and probably complex, mechanisms at the base of that phenomenon.

APPENDIX

BRAIN RESEARCH REVIEWS 55 (2007) 144–154

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BRAIN RESEARCH REVIEWS

Review

Mesolimbic dopamine and cortico-accumbens glutamate afferents as major targets for the regulation of the ventral striato-pallidal GABA pathways by neurotensin peptides

Luca Ferraro[a], *Maria C. Tomasini*[a], *Kjell Fuxe*[b], *Luigi F. Agnati*[c], *Roberta Mazza*[a], *Sergio Tanganelli*[a,*], *Tiziana Antonelli*[a]

[a]*Department of Clinical and Experimental Medicine, Section of Pharmacology, University of Ferrara, Via Fossato di Mortara 17-19, 44100 Ferrara, Italy*
[b]*Department of Neuroscience, Division of Cellular and Molecular Neurochemistry, Karolinska Institutet, S-171 77 Stockholm, Sweden*
[c]*Department of Biomedical Sciences, Section of Physiology, University of Modena and Reggio Emilia, 41100 Modena, Italy*

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ABSTRACT

The tridecapeptide neurotensin (NT) acts in the mammalian brain as a primary neurotransmitter or neuromodulator of classical neurotransmitters. Morphological and functional *in vitro* and *in vivo* studies have demonstrated the existence of close interactions between NT and dopamine both in limbic and in striatal brain regions. Additionally, biochemical and neurochemical evidence indicates that in these brain regions NT plays also a crucial role in the regulation of the aminoacidergic signalling. It is suggested that in the nucleus accumbens the regulation of prejunctional dopaminergic transmission induced by NT may be primarily due to indirect mechanism(s) involving mediation via the aminoacidergic neuronal systems with increased glutamate release followed by increased GABA release in the nucleus accumbens rather than a direct action of the peptide on accumbens dopaminergic terminals. The neurochemical profile of action of NT in the control of the pattern of dopamine, glutamate and GABA release in the nucleus accumbens differs to a substantial degree from that shown by the peptide in the dorsal striatum. The neuromodulatory NT mechanisms in the regulation of the ventral striato-pallidal GABA pathways are discussed and their relevance for schizophrenia is underlined.

Contents

* *Corresponding author*. Fax: +39 0532 291205.
E-mail address: tgs@unife.it (S. Tanganelli).
Abbreviations: DA, Dopamine; NT, Neurotensin; VTA, Ventral tegmental area

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1. Introduction

In the mammalian brain the dopaminergic system plays a pivotal role in the control and modulation of movement, cognition, memory as well as affective and reward behaviours (Horvitz, 2000; Nieoullon, 2002; Wise, 2004; 2005; Nestler and Carlezon, 2006; Fuxe et al., this special issue). The substantial neurophysiological and neuropharmacological heterogeneity shown by dopaminergic neurons may originate not only from the diversity of their intrinsic properties such as different receptor subtypes and their anatomical localization but also from extrinsic factors as the vast combinatorial possibilities in terms of neuronal connections (White, 1996; Missale et al., 1998; Saleem et al., 2000). Thus, the large difference in term of afferent and efferent connections of dopamine (DA) neurons compared with other transmitter-identified neurons such as glutamate, acetylcholine, gamma-aminobutyric acid (GABA), and serotonin neurons may explain the diverse function and the complexity of the dopaminergic neural networks. The two main ascending midbrain dopaminergic neurons have their cell bodies located in two distinct nuclei of the mesencephalon: the substantia nigra pars compacta (SNc) (forming the so-called nigrostriatal pathway) (Gerfen, 1992; *see* Fuxe et al., 2006) and the ventral tegmental area (VTA). Neurons of the VTA primarily project to the ventral striatum (nucleus accumbens and olfactory tubercle) as part of the so-called mesolimbic system and to the prefrontal cortex (the so-called mesocortical system) but additional ascending DA connections are established with the septal area, amygdala and hippocampus (Hyman and Malenka, 2001; Everitt and Wolf, 2002; Kalivas, 2004). Such an anatomical organization not only supports the physiological relevance of these pathways but also the belief that a disruption in the activity of these central DA-containing neurons is associated with the expression of neurological and psychiatric disorders (Fibiger, 1995; Nestler and Carlezon, 2006). In particular, the degeneration of DA cells in the SNc and the consequential loss of DA innervation of the striatum is considered the cornerstone of the DA hypothesis in Parkinson's disease (Fuxe et al., 2006), whereas, in a simplistic theory the hyperactivity of DA transmission in the mesolimbic system has been linked to the development of schizophrenia, psychosis, dyskinesia, and dystonia (Tzschentke, 2001; Adell and Artigas, 2004; Fuxe et al., this special issue). In addition, the euphoric and rewarding behaviours evoked by drugs of addiction, are mediated by alteration in the functional activity of this dopaminergic system (Wise, 2005; Fuxe et al. this special issue). Thus, based on the existence of a relationship between the abnormal activity of dopaminergic systems and some neurological and neuropsychiatric human disorders, the DA-replacement therapy and treatment with DA D2 receptor antagonists have become the main therapies for Parkinson's disease and schizophrenia, respectively. Unfortunately, current treatments of these disorders are still symptomatic and a preventive approach is still lacking.

The mesolimbic DA neurons are richly interconnected with other neuronal systems forming a complex and composite circuitry. In this circuitry the nucleus accumbens may be considered as one of the main strategic interface for the integration of limbic signals since it receives excitatory glutamatergic projections from the prefrontal cortex, the basolateral amygdala and the hippocampus (Brog et al., 1993; Pennartz et al., 1994; Groenewegen et al., 1999). It sends emotional information back to the prefrontal cortex via a circuit involving the so-called ventral striato-pallidal GABA pathway, the ventral pallidal GABA projection to the mediodorsal thalamic nucleus and the mediodorsal thalamic nucleus-prefrontal glutamate projection (Zahm and Heimer, 1993; Kalivas, 1993; Haber et al., 1995; Zahm et al., 1996; Groenewegen et al., 1999). Nucleus accumbens also sends projections to the VTA, which gives rise to the mesolimbic and mesocortical DA projections and to the substantia nigra (Groenewegen et al., 1999; Usuda et al., 1998; Dallvechia-Adams et al., 2001) in this way also influencing motor functions.

DA receptors are located on postjunctional GABAergic medium-sized spiny neurons and on glutamate prefrontal-accumbens afferents in the nucleus accumbens projecting to the ventral pallidum (Groenewegen et al., 1991; Heimer et al., 1993; O'Connor, 2001) and mesolimbic DA afferents exert a tonic inhibitory regulation of ventral striato-pallidal GABA transmission. Thus, the blockade of accumbens D2 like receptors and the consequent activation of ventral striato-pallidal GABA transmission appears to be a common effect of antipsychotic agents leading to the restoration of the excitatory

drive of the thalamo-prefrontal glutamate projection which seems to be reduced in schizophrenic patients (Carlsson et al., 1999; O'Connor, 2001). On the other hand, alterations in the functional activity of the convergent prefrontal glutamatergic excitatory afferents arriving into the nucleus accumbens and/or dysfunctions in the inhibitory accumbens GABAergic efferent pathways themselves could also underlie the behavioural and emotional impairments characteristic of schizophrenia (O'Donnell and Grace, 1998) and the understanding of such impairments may be fundamental for the pharmacotherapy of the disease. As a consequence of this possibility, it could be suggested that changes in mesolimbic DA function may represent a compensatory response to or a consequence of a primary defect occurring in some of the other neuronal systems converging or departing from the nucleus accumbens.

To make the scenario more complex there exists evidence indicating that in these brain areas DA is often co-localized in discrete neuron populations with neuropeptides, which are known to play an important role in the modulation of classical neurotransmitter function. In this context, the discovery that the tridecapeptide neurotensin (NT) is co-localized with DA in mesocortical neurons but not in nigrostriatal and mesolimbic DA neurons suggests that NT might play a special role in the regulation of meso-cortical DA transmission (Kalivas and Miller, 1984; Seroogy et al., 1987; Studler et al., 1988). Thus, the complex interactions between NT and DA which take place in these brain regions including NT/D2 receptor–receptor interactions have received increasing attention as to their involvement in the pathophysiological consequences of disturbed mesolimbic DA neuron function and as potential targets for novel anti-schizophrenic therapy (*see* review Caceda et al., 2006; Antonelli et al., submitted). In this context, a potential antipsychotic profile for NT agonists has been proposed on the basis of their ability to block postjunctional D2 mediated DA transmission in the nucleus accumbens (Caceda et al., 2006) without increasing DA release (Tanganelli et al., 1994). In view of the above it may be concluded that a primary intrinsic abnormality in the mesolimbic dopaminergic transmission is not the only underlying factor for limbic dysfunction in schizophrenia but a concurrent dysfunction of other neuronal systems located within the nucleus accumbens and/or prefrontal cortex having close interactions with the dopaminergic mesolimbic system may also have a critical role in the pathophysiology of this neuropsychiatric disorder.

In the following sections of this review, we will deal with the biochemical and neurochemical results that have increased our understanding of the role NT plays in the control of dopaminergic and aminoacidergic signalling particularly in the nucleus accumbens and its dysfunction in psychiatric disorders especially schizophrenia. A comparison will be made on its role in the local circuits in the nucleus accumbens versus those of the striatum. The review begins with a short overview of the NT mechanisms.

2. Neurotensin (NT)

NT, is a gut–brain tridecapeptide which was firstly isolated from the bovine hypothalamus by Carraway and Leeman in 1973 and subsequently from bovine intestine (Kitabgi et al., 1976).

2.1. *Biochemistry of neurotensin*

NT and its structurally related active analogue neuromedin N (NN) are synthesized within neurons throughout the CNS as part of a larger inactive precursor of 169 amino acid residues. The precursor molecule, containing both NT and NN near the C-terminus, undergoes a differential tissue-specific cleavage at its dibasic sites by proprotein convertase. Once processed as an active peptide in neurons, NT is stored in dense core vesicles and released in a calcium-dependent manner. The physiological inactivation of NT is operated by endopeptidases belonging to the family of metallopeptidases in particular by the endopeptidase 24.16 which is expressed ubiquitously (Kislauskis et al., 1988; Villeneuve et al., 2000; Kitabgi, 2006). Another mechanism that produces an inactivation of NT transmission is the process of NT internalization (Mazella and Vincent, 2006b). NT is widely distributed in mammalian brain (Cooper et al., 1981; Emson et al., 1982) and it has been demonstrated that high levels of the peptide are present in the hypothalamus, amygdala, bed nucleus of the stria terminalis, lateral septum, nucleus accumbens, caudate–putamen and ventral tegmental area (Tyler-McMahon et al., 2000; Geisler et al., 2006). Such a distribution of the peptide matches the distribution of the NT receptors in the brain (Vincent et al., 1999; Geisler et al., 2006).

2.2. *Neurotensin receptor subtypes and their location*

The biological actions of NT are initiated by binding to three different receptor subtypes NTS1, NTS2 and NTS3 (Geisler et al., 2006; Mazella and Vincent, 2006a). NTS1 and NTS2 receptors belong to the family of G-protein-coupled receptors (Pelaprat, 2006) mediating activation of phospholipase C, production of inositol phosphate and mobilization of intracellular calcium (Vincent et al., 1999). However, the signal transduction mechanism associated with NTS2 is still controversial (Mazella and Vincent, 2006a). NTS1 and NTS2 are also differentiated on the basis of their affinity for NT and their sensitivity to levocabastine, an antihistaminic compound: NTS1 is levocabastine-insensitive with a high affinity for NT whereas NTS2 is levocabastine-sensitive with a low affinity for NT (Tanaka et al., 1990; Vita et al., 1998; Vincent et al., 1999; St-Gelais et al., 2006). NTS3 belongs to the family of sorting receptors characterized by the presence of a luminal/extracellular region containing a cysteine-rich domain and a single transmembrane domain preceding a short intracellular tail bearing signals for rapid internalization. Its function is still undefined (Hermans and Maloteaux, 1998; Vincent et al., 1999; Sarret et al., 2003; Mazella and Vincent, 2006a). The majority of the central effects of NT appear to be exerted through the high affinity NTS1 that has been the most extensively studied of the three NT receptor subtypes.

2.3. *Chemical neuroanatomy*

Complementary autoradiographic and immunohistochemical techniques as well as a more recent electron microscopic

analysis performed with a polyclonal antibody recognising the amino-terminal of the NTS1 (Fassio et al., 2000) provide similar results in relation to the distribution of NTS1 in the rat CNS. Thus, a high density of NTS1 has been shown within the VTA, the substantia nigra, the hypothalamus, the central nucleus of the amygdala with somewhat reduced densities in the nucleus accumbens, septum, striatum and globus pallidus (Boudin et al., 1996; Alexander and Leeman, 1998; Binder et al., 2001). At the electron microscopic level immunoreactive NTS1 receptors has been found both in axonal buttons, dendrites and spines in the basal forebrain indicating that NT may act both at the nerve terminal (Hetier et al., 1988; Faggin et al., 1990; Heaulme et al., 1997; Legault et al., 2002) and at the somatodendritic level (Seutin et al., 1989; Jiang et al., 1994; Werkman et al., 2000). There exist findings indicating high densities of NT immunoreactive nerve terminals nearby dopaminergic cell bodies within the substantia nigra and VTA where the peptide is also co-localized within a distinct group of dopaminergic neurons projecting to the cerebral cortex (Hökfelt et al., 1984; Berger et al., 1992). These findings suggest that NT transmission is also relevant in regulating dopaminergic neuronal pathways involved in schizophrenia (Kinkead et al., 1999; Dobner, 2005; Caceda et al., 2006) and in reward (Romprè et al., 1992; Geisler et al., 2006) at the level of the midbrain DA cell bodies.

3. Neurochemical analysis of the neurotensin regulation of the local circuits of the nucleus accumbens

From a neurochemical and functional point of view evidence has accumulated that activation of somatodendritic NTS1 increases the firing rate of mesolimbic ad mesocortical dopaminergic neurons (Seutin et al., 1989; Werkman et al., 2000) most likely by increasing intracellular Ca^{2+} (St-Gelais et al., 2004) and reducing K^+ conductances (Mercuri et al., 1993; Farkas et al., 1997). The location of NT receptor at the presynaptic levels of the DA neurons is not fully elucidated since the data available in the literature are still controversial and inconsistent. For instance, Quirion et al. (1992) reported that the destruction of mesolimbic dopaminergic neurones following 6-hydroxydopamine induced lesions of the VTA only marginally reduces the number of NT binding sites in the nucleus accumbens and therefore suggested only a low density of NT receptors at the prejunctional level of the mesolimbic dopaminergic neurons. Accordingly, as recently reviewed by Binder et al. (2001), NTS1 receptors within the ventral striatum have been detected in medium spiny and aspiny GABAergic neurons and rarely on nerve terminals (Nicot et al., 1994; Boudin et al., 1996; Delle Donne et al., 1996; Alexander and Leeman, 1998). On the contrary, Pickel et al. (2001) revealed that throughout the nucleus accumbens, NTR-LI was present in axon terminals resembling both cortical glutamatergic and striatal dopaminergic afferents as well as in collaterals of inhibitory projection neurons. In view of the above it may be suggested that local application of NT in the nucleus accumbens regulates prejunctional dopaminergic transmission mainly via indirect mechanisms involving other neuronal systems (*see* next paragraph) rather than through a direct activation of the few NTS1 receptor located on accumbal dopaminergic terminals. As a consequence of the findings indicating that the location of NT receptors are not only expressed on neurons but also on astrocytes (Nouel et al., 1999; Trudeau, 2000) it could be suggested that under specific conditions of local treatments such as perfusion of NT via a microdialysis probe, the NT induced changes on extracellular dialysate levels of glutamate could be considered not only as a result of the activation of the neuron (synaptic release) but also of astrocytes (volume transmission). The term volume transmission describes the transfer of a signal in the extracellular space including the diffusion of chemical signals like transmitters and modulators in the extracellular fluid. Thus, the NT-induced release of glutamate from astrocytes into the extracellular compartment, may induce a concentration gradient leading to diffusion of the aminoacid for relatively long distances with activation of extrasynaptic glutamate receptors on the target cells (Agnati et al., 1986; Timmerman and Westerink, 1997; Del Arco et al., 2003; Fillenz, 2005; Agnati et al., 2006). In contrast, the changes induced by the local perfusion of NT on dopamine extracellular levels are mainly linked to the enhancement of neuronal DA release from DA terminals via activation of NTS1 receptors on these terminals mediating mainly volume transmission effects but also synaptic effects detected in the extracellular fluid via DA synaptic spillover.

3.1. *Neurochemical evidence for a GABA-mediated regulation of dopamine transmission in the nucleus accumbens by neurotensin*

In the nucleus accumbens local activation of NTS1 by NT in low (10 nM) nanomolar concentrations induces an enhancement of GABA outflow which is tetrodotoxin sensitive, calcium dependent and potentiated in presence of an inhibition of the 3.4.24.15. and 3.4.24.16 metalloendopeptidases (Tanganelli et al., 1994; O'Connor, 2001). Such a facilitation of GABAergic signalling is associated with a concomitant and significant reduction of the accumbal DA release. The involvement of a GABAergic mechanism in the NT-induced reduction of accumbal DA outflow has been proven by the evidence that blockade of the GABA-A receptor in the nucleus accumbens by local perfusion with bicuculline counteracts the inhibitory effects of NT on DA outflow without affecting the enhancement of GABA release (Tanganelli et al., 1994). In view of the demonstration by electron microscopic immunolabelling analysis that NTS1 are also located at axon terminals resembling dopaminergic and cortical glutamatergic afferents in the nucleus accumbens (Pickel et al., 2001) it may be suggested that NT, perfused into the nucleus accumbens, by activating NTS1 receptors mainly located on accumbal glutamate terminals induces an enhancement of glutamate outflow. Such an increase can then activate the inhibitory GABAergic signalling of dendrites and collaterals of the ventral striato-pallidal GABA pathway that could be responsible for the significant reduction of DA release observed in the nucleus accumbens (Tanganelli et al., 1994).

In support of this hypothesis are *in vitro* and *in vivo* data indicating that NT enhances endogenous glutamate outflow

in different brain regions (Sanz et al., 1993; Saleh et al., 1997; Ferraro et al., 1997, 1998, 2000, 2001; Matsuyama et al., 2003; Chen et al., 2006). In addition to this mechanism, it cannot be excluded that the inhibition of accumbal DA outflow induced by intra-accumbens NT perfusion may be also in part due to a direct activation of the NTS1 located on GABAergic neurons (Binder et al., 2001) that raises GABA extracellular levels and induces an inhibition of DA outflow.

3.2. *Differential regulation by neurotensin of local circuits in the nucleus accumbens with respect to the dorsal striatum*

It is worth noting that the neurochemical profile of action of NT in the control of DA and GABA signalling in the nucleus accumbens differs from that shown by the peptide into the dorsal striatum. In fact, by using the same methodological approach (*in vivo* microdialysis) it has been possible to demonstrate that, in contrast to that observed in the nucleus accumbens, the intrastriatal perfusion of NT is associated with an increase of local DA levels at 1 μM concentration and never causes a direct or GABA-mediated reduction of striatal DA levels (Tanganelli et al., 1989; Fuxe et al., 1992a; Ferraro et al., 1997). Thus, it might be suggested that in the dorsal striatum, differently from that observed in the nucleus accumbens, NT increases dopaminergic signalling mainly via the activation of a relatively high density of NTS1 receptors located on striatal DA terminals (Li et al., 1995; Tanganelli et al., 1994).

4. Pre and postjunctional antagonistic NTS1/D2 receptor–receptor interactions as a major mechanism in the NT regulation of the local circuits of the ventral and dorsal striato-pallidal GABA neurons

4.1. *Nucleus accumbens*

Several and diverse mechanisms may underlie the effects of NT in the regulation of the mesolimbic dopaminergic neurons and their pre and postsynaptic mechanisms. One of these possible mechanisms may be triggered by an antagonistic action of the peptide on DA D_2 receptor function at the pre and postjunctional level (Agnati et al., 1983; Fuxe et al., 1992b). In particular, Fuxe and Agnati (1985) have developed the concept that a heterostatic receptor–receptor interaction may be the main mechanism underlying the regulation of D2 receptor function by neuropeptides. Concerning NT such a mechanism of action is supported by biochemical receptor binding and autoradiographic studies demonstrating that NT selectively reduces the affinity of the DA D2-like agonist [3H]N-propyl-norapomorphine binding sites (Agnati et al., 1983; von Euler et al., 1990a,b; 1991; Li et al., 1995). In particular, NT pre-treatment increased the K_d value of the DA D2 receptor agonist by a preferential enhancement of the dissociation rate from the high affinity form of the D2 receptor without affecting the B_{max} value. This modulation was observed both in subcortical limbic brain regions as well as in the striatum and was stronger in sections than in membrane preparations indicating the requirement of intact cell membranes for full action (von Euler et al., 1989; Fuxe et al., 1992a,b; Tanganelli et al., 1993; Li et al., 1995). This effect appears to be specific for D_2-type receptor agonist binding sites since at these low nanomolar concentrations NT does not affect D_1 receptor agonist or antagonist binding sites (von Euler et al., 1991; Fuxe et al., 1995).

In line with the antagonistic NTS1/D2 receptor–receptor interactions, observed in membrane preparations and striatal sections, *in vivo* microdialysis studies demonstrated that intra-accumbens application of NT at low (1 nM) concentration, by itself ineffective both on endogenous extracellular DA and GABA levels, when co-perfused with the D2-like receptor agonist pergolide significantly counteracted the inhibitory effect induced by the D2 receptor agonist on GABA outflow but was completely ineffective on the pergolide-induced inhibition of DA outflow that remained unchanged (Li et al., 1995). These results provide functional evidence that in the nucleus accumbens the antagonistic action of NT observed, at low nanomolar concentration, on D2 receptor function takes place primarily at the post-junctional level. Thus, in line with the functional findings reported above and the location of D2 and NTS1 receptors on cortico-accumbens glutamatergic terminals it may be concluded that in the nucleus accumbens there exists a dominance of the postjunctional antagonistic NTS1/D2 receptor–receptor interactions on the cortico-accumbens glutamate terminals over the weak antagonistic NTS1/D2 receptor–receptor interaction on the accumbens DA terminals resulting in the release of glutamate which causes GABA release (Fig. 1). However, it cannot be excluded that the few NTS1 located on the ventral striato-pallidal GABAergic neurons can contribute to the release of GABA from these neurons, where also antagonistic NTS1/D2 receptor interactions may exist (Binder et al., 2001).

4.2. *Striatum*

Interestingly, the functional consequences of the antagonistic NTS1/D2 receptor–receptor interaction which takes place in the nucleus accumbens differs from those observed in the striatum. Intrastriatal perfusion with the preferential DA D_2 receptor agonist pergolide, inhibits striatal DA and GABA outflow. The intrastriatal co-perfusion of a low nanomolar concentration of NT, by itself ineffective, in combination with pergolide fully abolished the inhibitory effects of the D_2 receptor agonist on DA and GABA release and this action was fully blocked by the presence of the NTS1 antagonist SR48692 (Gully et al., 1993) in the perfusate medium. These results provide a functional *in vivo* correlate to the binding data and suggest the existence of antagonistic NTS1/D_2 receptor–receptor interactions which mainly take place on cortico-striatal glutamate terminals and on the striatal DA terminals according to the chemical neuroanatomical findings. However, the involvement of the few NTS1 located postsynaptically on the striatopallidal GABA neurons can again not be excluded, since they also probably form heteromers with the D2 receptors and inhibit their signalling (Antonelli et al., submitted).

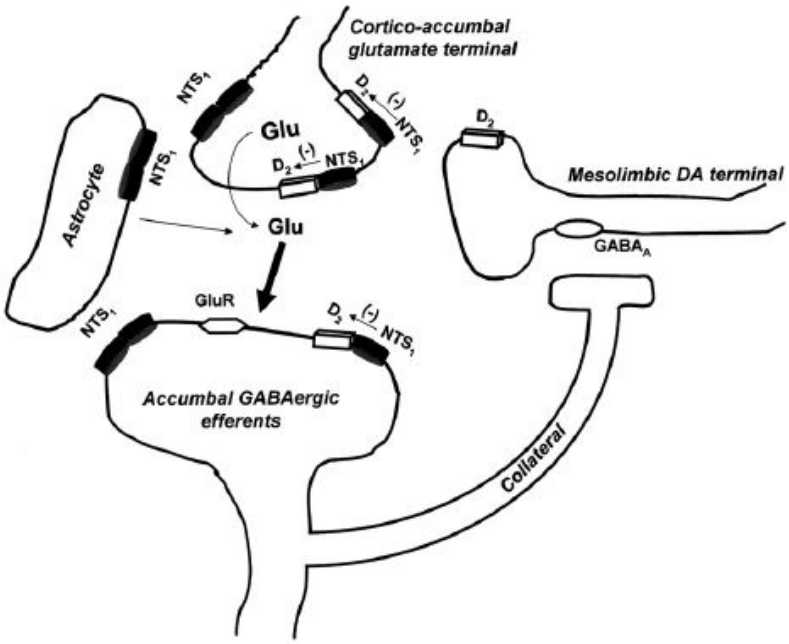

Fig. 1 – Main location of the NTS1/D2 heteromers mediating the NTS1/D2 receptor–receptor interactions in the control of the ventral striato-pallidal GABA pathway from the nucleus accumbens. In the nucleus accumbens there exists a dominance of the postjunctional antagonistic NTS1/D2 receptor–receptor interactions on the cortico-accumbens glutamate terminals over the weak antagonistic NTS1/D2 receptor–receptor interaction on the accumbens DA terminals (not shown). The intra-accumbens perfusion of NT antagonizes the inhibitory dopamine D2 tone on glutamatergic terminals via a NTS1/D2 receptor–receptor interaction thus inducing an enhancement of glutamate outflow. Such an increase can then activate the inhibitory GABAergic signalling that could be responsible, via a collateral, for the significant reduction of DA release observed in the nucleus accumbens (Tanganelli et al., 1994). However, it cannot be excluded a direct involvement of NTS1 located on GABAergic neurons (Binder et al., 2001). The two D2/NTS1 heteromers on the glutamate terminals are to show that this is their major location, since only few are found on the striato-pallidal GABA neurons. NTS1 alone without D2 could be a monomer or a homomer which is true also for D2 when not present in a heteromer. Finally, although there is no direct evidence today for NTS1 modulating astroglia glutamate release, the possibility for astrocytes as part of the neurochemical substrates that give rise to the release of glutamate is also represented.

4.3. *NTS1/D2 receptor–receptor interaction differently regulates dopamine and GABA levels in the nucleus accumbens with respect to the striatum*

The above neurochemical and biochemical findings indicate that the neuromodulatory actions of NT are produced by the interference of the peptide with the inhibitory D2 receptor signalling, postulated to take place mainly via the existence of a NTS1/D2 receptor heteromeric complex located on the plasma membrane. In view of these results it may be suggested that in the dorsal striatum most of the neuromodulatory effects of NT take place on striatal DA terminals and on cortico-striatal glutamate terminals with respect to the cell body or dendrites of GABA neurons.

In contrast, in the nucleus accumbens the regulatory actions of NT are due to an antagonistic NTS1/D2 receptor–receptor interaction occurring on the cortico-accumbens glutamate terminals and possibly at the postsynaptic level on the efferent GABAergic neurons with only weak if any antagonistic NTS1/D2 autoreceptor receptor interactions on the accumbens DA terminals. The involvement of glutamatergic transmission in these effects is supported by microdialysis studies demonstrating that NT plays a crucial role in the regulation of glutamate levels in the basal ganglia as well as in the cerebral cortex (Chapman and See, 1996; Ferraro et al., 1997, 1998, 2000, 2001; Petrie et al., 2005; Chen et al., 2006). Thus, it may be postulated that activation of NTS1 receptors located on cortico-striatal and cortico-accumbal glutamate terminals may lead to an increase of glutamate outflow in the striatum and nucleus accumbens that counteracts the pergolide induced-inhibition of GABA signalling (Wagstaff et al., 1996; Binder et al., 2001; Dobner et al., 2003).

5. Possible relevance for the understanding of the role of neurotensin in psychostimulant drug actions and in schizophrenia

The mesolimbic and mesocortical DA pathways are highly implicated in the pathogenesis of schizophrenia and in the psychomotor stimulant and reinforcing effects of drugs, such as D-amphetamine and cocaine. Because of the well known interactions between NT and the mesolimbic and mesocortical DA systems, NT signalling appears to be one of the

specific neurochemical mechanisms for the rewarding activity of cocaine and other drugs of abuse (McBride et al., 1999; Richelson et al., 2003; Fredrickson et al., 2005; Lopak and Erb, 2005) and have also been implicated in the pathogenesis of schizophrenia and in the mechanism of action of antipsychotic drugs (Kinkead and Nemeroff, 2002, 2006; Dobner et al., 2003; St-Gelais et al., 2004; Dobner, 2005; Caceda et al., 2006; Boules et al., 2005; 2007; Geisler and Zahm, 2006). Since the potential involvement of NT in schizophrenia and psychostimulant actions has been recently reviewed, here the functional paradox of neuroleptic action versus psychostimulant effects of NT will be discussed.

Substantial evidence shows that NT is involved in amphetamine and cocaine sensitization and the NTS1 antagonist delays, diminishes or blocks the development of psychostimulant sensitization depending on the treatment protocol used (Costa et al., 2001; Horger et al., 1994; Betancur et al., 2001; Panayi et al., 2005). Furthermore, central NT receptors upon activation can reinstate cocaine seeking in rodents (Lopak and Erb, 2005) giving further indications for a role of NT receptors also in the altered neuroplasticity that underlies development of cocaine addiction. To understand the NT receptor mechanisms involved it is important to underline that in contrast to the case after treatment with typical antipsychotic drugs, psychostimulants increase NT levels mainly in subpopulations of the striato-nigral GABA pathways (Merchant, 1994) regulated primarily by the D1 receptors. Unlike many D2 receptors (*see* above) D1 receptors are not antagonistically regulated by NTS1 and also mediate rewarding actions of psychostimulants. The NTS1 mechanism appears essential for the c-*fos* expression and thus activation of subpopulations of the striato-nigral GABA pathway (Fadel et al., 2006) that may be part of the reward pathways, especially when originating from the nucleus accumbens.

One essential mechanism may be that the psychostimulant induced NT synthesis and release in the cell body-dendritic regions of these nerve cells allows NT to diffuse as a volume transmission signal to cause inhibition of D2 autoreceptor signalling via the antagonistic NTS1/D2 interaction in the striatal and accumbal DA terminals leading to increased DA release associated with increased activation of the D1 receptors favouring activation of these reward pathways. Another essential mechanism may be the ability of the diffusing NT to activate NTS1 on the close glutamate terminals in the same local circuits increasing glutamate release which also may contribute to the activation of certain striato-nigral and accumbal-VTA GABA pathways participating in the reward mechanisms activated by psychostimulants. In this way it becomes possible to understand how NT mechanisms contribute to the development of psychostimulant sensitization and possibly to cocaine addiction. Similar NTS1 mechanisms may also exist in the VTA leading to the increased activation of the mesolimbic DA neurons which is a well-known reward system. Thus, also here NTS1 activation may antagonize DA D2 autoreceptor functions and increase glutamate drive. Long-term changes in gene transcription may in this way be triggered and cause plastic changes in the meso-limbic DA reward systems and thus to sensitization and addiction development.

A potential antipsychotic profile for NT has also been proposed on the basis, among others, of its effects on neuronal elements in the nucleus accumbens. In fact, as previously reported, intra-accumbens perfusion with NT (10 nM) increases GABA release in the nucleus accumbens and in turn reduced local extracellular DA levels (Fig. 1). It is worth noting that the behavioural effects of systemically administered NTS1 agonists resemble those of direct intra-accumbens NT application (Caceda et al., 2006), suggesting an involvement of the activation of ventral striato-pallidal GABA signalling in the neuroleptic like effects of NT agonists. Interestingly, antipsychotics also increase GABA release in the accumbens (Drew et al., 1990; Ferrè et al., 1994; Osborne et al., 1994) and the activation of ventral striato-pallidal GABA transmission appears to be a common effect of these drugs. In addition, typical and atypical antipsychotic drugs increase NT levels in the nucleus accumbens (*see* Frey et al., 1986) which strengthens the hypothesis raised by group of Nemeroff and colleagues that increased NT transmission in the limbic system could be involved the therapeutic effects of antipsychotic drugs. Converging evidence from studies on the pathophysiology of schizophrenia links increased mesolimbic DA neuronal activity (i.e. hyperdopaminergia) to reduced thalamo-cortical glutamate (i.e. hypoglutamatergia) drive (Carlsson et al., 1999; O'Connor, 2001). It can therefore be speculated that NT as a volume transmission signal exerts its antipsychotic action by increasing ventral striato-pallidal GABA transmission, mainly through a postjunctional antagonistic NTS1/D2 receptor–receptor interaction on the cortico-accumbens glutamate terminals (*see* above), thus restoring the excitatory drive of the thalamo-prefrontal glutamate projection which seems to be reduced in schizophrenic patients (Carlsson et al., 1999; O'Connor, 2001).

From this biochemical and neurochemical overview it appears that the observed anti-schizophrenia like activity of NT could be in part related to the fact that NT, at low concentrations in the nucleus accumbens by selectively antagonizing the postjunctional inhibitory DA D_2 receptor function on the cortico-accumbens glutamate terminals, can increase glutamate release. This assists in the triggering of an activation of the ventral striato-pallidal GABA pathway causing restoration of the mediodorsal thalamic nucleus glutamate drive to the prefrontal cortex, an effect which is common to antipsychotic drugs and related to their ability to block inhibitory DA D_2 like receptors located on this ventral striato-pallidal GABA pathway. These neurochemical data are in line with the hypothesis raised by Nemeroff and colleagues (Nemeroff, 1980; Nemeroff and Cain, 1985; Kinkead and Nemeroff, 2002, 2006) that an enhancement of NT signalling in the nucleus accumbens induced by the DA D_2 receptor blocking actions of antipsychotic drugs could contribute to the therapeutic effects of such drugs and give a new understanding of the NT mechanisms involved at the molecular level.

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Neurotensin Receptor Involvement in the Rise of Extracellular Glutamate Levels and Apoptotic Nerve Cell Death in Primary Cortical Cultures after Oxygen and Glucose Deprivation

Tiziana Antonelli[1], Maria C. Tomasini[1], Jacqueline Fournier[2], Roberta Mazza[1], Sergio Tanganelli[1], Stefania Pirondi[3], Kjell Fuxe[4] and Ferraro Luca[1]

[1]Department of Clinical and Experimental Medicine, Pharmacology Section, University of Ferrara, 44100 Ferrara, Italy, [2]Sanofi-Aventis Recherche, 40064 Toulose, France, [3]Department of Veterinary Morphophysiology and Animal Production, University of Bologna, S-171 77 Bologna, Italy and [4]Department of Neuroscience, Karolinska Institute, 31071 Stockholm, Sweden

In view of the ability of neurotensin (NT) to increase glutamate release, the role of NT receptor mechanisms in oxygen–glucose deprivation (OGD)–induced neuronal degeneration in cortical cultures has been evaluated by measuring lactate dehydrogenase (LDH) levels, mitochondrial dehydrogenase activity with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide levels, and microtubule-associated protein 2 (MAP2) immunoreactivity. Apoptotic nerve cell death was analyzed measuring chromatin condensation with Hoechst 33258, annexin V staining, and caspase-3 activity. Furthermore, the involvement of glutamate excitotoxicity in the neurodegeneration-enhancing actions of NT was analyzed by measurement of extracellular glutamate levels. 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 neurotensin receptor 1 (NTR1) antagonist SR48692. Unexpectedly, the antagonist at 100 nM counteracted not only the NT effects but also some OGD-induced biochemical and morphological alterations. These results suggest that NTR1 receptors may participate in neurodegenerative events induced by OGD in cortical cultures, used as an in vitro model of cortical ischemia. The NTR1 receptor antagonists could provide a new tool to explore the clinical possibilities and thus to move from chemical compound to effective drug.

Keywords: cortical cell cultures, ischemia, lactate dehydrogenase, MAP2 immunoreactivity, neurotensin receptor antagonist

Introduction

The brain requires a continuous supply of oxygen and glucose to maintain normal function and viability. The idea that hypoxic-ischemic brain damage can be explained by extracellular accumulation of glutamate and overstimulation of glutamate receptors is appealing. In particular, N-methyl-D-aspartate (NMDA) receptors (Goldberg and Choi 1993; Zipfel et al. 2000) may contribute to hypoxic–ischemic neuronal injury, and in fact, treatment with glutamate antagonists can limit hypoxic-ischemic brain damage (Mueller et al. 1999; Johnston et al. 2001; Culmsee et al. 2004). Many aspects of ischemic neurodegeneration have been demonstrated in animal models. However, the ischemic process represents a complex environment for the dissection of the cellular and molecular mechanisms involved in ischemic neurodegeneration (Arundine and Tymianski 2004; Young et al. 2004). Cell culture systems could therefore represent a more defined microenvironment and a simple experimental model to study some aspects of ischemic-induced neurodegeneration (Choi et al. 1990). It has been demonstrated in cultured cortical neurons that glutamate excitotoxicity via NMDA receptors induces apoptosis or necrosis depending on the intensity of the insult. Indeed, a mild glutamate insult leads to an apoptotic cell death, whereas an intense glutamate insult induces predominantly a necrotic process (Cheung et al. 1998). Therefore, endogenous compounds able to modulate glutamatergic transmission may interfere with glutamate-induced cell death.

In this context, it has been shown that the tridecapeptide neurotensin (NT) significantly enhances glutamatergic signaling in both in vitro (Ferraro et al. 2000; Matsuyama et al. 2003; Chen et al. 2006) and in vivo (Ferraro et al. 1995, 1998, 2001) studies. These findings suggest a reinforcing action of NT on several functions exerted by glutamate in the central nervous system, in particular on the glutamate-mediated excitotoxicity (Antonelli et al. 2007). An involvement of NT in modulating glutamate excitotoxicity has recently been demonstrated in primary cultures of mesencephalic dopamine (Antonelli et al. 2002) and cortical (Antonelli et al. 2004) neurons. Nevertheless, the effects of NT on glutamate transmission in the cerebral cortex, an important cerebral area damaged by pathological events like ischemia, are still undefined. In this context, an increase of NT levels in rat cerebral cortical areas has been demonstrated following focal ischemia by Allen et al. (1995), suggesting a possible involvement of NT in ischemic brain damage. Furthermore, in vivo experiments provide evidence that NT-induced hypothermia improves neurologic outcome and reduces infarct volume after hypoxic ischemia (Katz et al. 2004) and middle cerebral ischemia (Torup et al. 2003), respectively.

In view of the neuroprotection caused by NT-induced hypothermia (Kokko et al. 2005) in in vivo models, the aim of the present study was to investigate under normothermic conditions the role of neurotensin receptor 1 (NTR1) in nerve cell death and endogenous extracellular glutamate levels after oxygen-glucose deprivation (OGD) in cerebral cortex cell cultures using a selective NTR1 antagonist SR48692. This in vitro approach may provide a more defined microenvironment where the presence of a vascular compartment and changes in temperature do not influence the results obtained.

Materials and Methods

Primary Cultures of Rat Cortical Neurons

Primary cultures of cortical neurons were prepared from embryonic day 18 Sprague–Dawley rats. Removed cortices were dissected free of meninges and dissociated in 0.025% (w/v) trypsin. The tissue fragments were dissociated mechanically by repeated gentle pipetting through wide- and narrow-bore fire-polished Pasteur pipettes in culture medium (Neurobasal medium with supplements of 0.1 mM glutamine,

10 μg/ml gentamicina, and 2% B27). The cells were counted and then plated on poly-L-lysine (5 μg/ml)-coated dishes at a density of 2.5×10^6 cells per dish and on poly-L-lysine (5 μg/ml)-coated multiwells (24 wells) at a density of 200 000 cells per well. In the dishes used for Hoechst 33258 nuclear staining, annexin V staining, and microtubule-associated protein 2 (MAP2) immunocytochemistry (see below), the cells were plated on poly-L-lysine (50 μg/ml)-coated glass coverslips. Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cytosine arabinoside (1 μM) was added at 5 days in vitro (DIV) to prevent glial cell proliferation. The cultures were maintained for 8 DIV before experiments.

OGD Exposure

The cultures were exposed to a transient OGD. To this purpose, in the OGD group, the culture medium was replaced with a glucose-free Krebs-Ringer bicarbonate buffer that had previously been saturated with 95% N_2/5% CO_2 and heated to 37 °C. The cultures were then put into an anaerobic incubator (pO_2 < 2 mm Hg) with an atmosphere of 95% N_2 and 5% CO_2 and 98% humidity at 37 °C for 60 min. OGD was terminated by removing the cultures from the anaerobic incubator, by replacing the exposure buffer with oxygenated Krebs–Ringer bicarbonate buffer containing glucose and returning the cultures to the incubator under normoxic conditions. In control group, not submitted to OGD, the cultures were exposed to oxygenated Krebs–Ringer bicarbonate buffer containing glucose and placed in a humidified atmosphere of 5% CO_2/95% air for 60 min. All the experiments were performed 24 h later.

Determination of Endogenous Extracellular Glutamate Levels

On the day of the experiment, the cells were rinsed twice by replacing the culture medium with Krebs-Ringer bicarbonate buffer (37 °C). Thereafter, 3 consecutive fractions were collected renewing this solution (400 μl). The first 2 samples, collected every 50 min (before OGD), have been used to assess basal endogenous glutamate levels, whereas the third fraction was collected 24 h later. When required, NT and the NT receptor antagonist SR48692 were added to the cultures using the following experimental protocol: NT was added 50 min prior to OGD and maintained in contact with cells during the OGD. The NT receptor antagonist SR48692 was added 20 min prior to NT and maintained in contact with cells during NT and OGD exposure and during the 24-h period after OGD. NT and SR48692 alone were also tested.

Endogenous glutamate levels have been quantified using a high-performance liquid chromatography/fluorimetric detection system, including a precolumn derivatization *o*-phthaldialdehyde reagent and a Chromsep 5 (C18) column. The mobile phase consisted of 0.1 M sodium acetate, 10% methanol, and 2.5% tetrahydrofuran, pH 6.5. The limit of detection for glutamate was 30 fmol per sample.

The effects of the treatments on endogenous extracellular glutamate levels during the third fraction were reported and expressed as percentage changes of basal values, as calculated by the means of the 2 fractions collected prior to treatment.

Lactate Dehydrogenase Levels

The neuronal death was quantitatively evaluated measuring the lactate dehydrogenase (LDH) levels in the extracellular fluid 24 h after OGD exposure using the Cytotoxicity Detection Kit LDH (Roche, Basel, Switzerland). It has been previously established that LDH release correlates linearly with the number of damaged neurons after toxic insult. Sensitivity was $0.2–2 \times 10^4$ cells per well. Background LDH levels were determined in control cell cultures not exposed to OGD and were subtracted from all experimental values. The LDH level corresponding to complete neuronal death was determined by assaying sister cultures exposed to 1 mM glutamate for 24 h. The LDH values were expressed as percentage of the value found with complete neuronal death (100%). NT and SR48692 effects on OGD-induced increase in LDH levels were evaluated by following the same procedure as described for endogenous glutamate experiments.

MTT Assay

The integrity of mitochondrial enzymes in viable neurons was determined with a colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) levels. In live cells, mitochondrial enzymes have the capacity to transform MTT into insoluble formazon. Sensitivity was about 2.5×10^4 cells per well. Twenty-four hours after OGD, cultures were incubated with MTT 5 mg/ml for 4 h at 37 °C. The formazon was dissolved in isopropanol with 1 M HCl and colorimetrically (absorbance at 570 nm) quantified. Neuronal viability corresponded to the value of the optical density read at 570 nm. The results were expressed as the percentage of neuronal viability measured in control cell cultures (100%). NT and SR48692 effects on OGD-induced change in mitochondrial enzymes activity were evaluated by following the same procedure as described for endogenous glutamate experiments.

Nuclear Staining with Hoechst 33258

Twenty-four hours after OGD exposure, cells were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS), and then incubated for 20 min at room temperature with Hoechst 33258 (1 μg/ml in PBS). After rinsing with PBS, coverslips were mounted on slides with a solution containing 50% glycerol in 0.044 M citrate, 0.111 M phosphate buffer, pH 5.5, and visualized under a fluorescence microscope. The percentage of cells showing chromatin condensation (fragmented nuclei) was quantified by counting ≥3000 cells under each experimental condition (5 randomly selected fields per well, 9–18 wells per condition per experiment, and 5 independent experiments). NT and SR48692 effects on OGD-induced increase of apoptotic nuclei were evaluated by following the same procedure as described for endogenous glutamate experiments.

Annexin V Staining

Annexin V staining was carried out using human annexin V–fluorescein isothiocyanate (FITC) kit (Bender MedSystems, Burlingame, CA) according to the manufacturer's instructions. Annexin V is a phospholipid-binding protein with high affinity for phosphatidyl serine. Annexin V staining was used to label phosphatidyl serine translocated to the outer membrane surface of several cell types undergoing apoptosis. Cells cultured on coverslips were washed twice with a buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ and then incubated with a solution of 5 μl annexin V–FITC and propidium iodide (1 μg/ml). Stained cells were examined at the fluorescence microscope. To quantify the number of apoptotic cells, the annexin-positive/propidium iodide–negative [AN(+)/PI(−)] immunoreactive cells were counted, and the data were expressed as percent of counted cells. NT and SR48692 effects on OGD-induced increase of annexin V staining were evaluated by following the same procedure as described for endogenous glutamate experiments.

Caspase-3 Activity

Caspase-3 activity was measured in lysates of cortical neurons using the caspACE colorimetric assay system (Promega, Madison, WI) following the instructions of manufacturer. The colorimetric substrate *N*-acetyl-Asp-Glu-Val-Asp (DEVD)-*p*-nitroaniline (Ac-DEVD-pNA) provided in the caspACE assay system is labeled with the chromophore *p*-nitroaniline that is released from the substrate upon cleavage by caspase-3. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405 nm. Briefly, neurons were lysed in ice-cold lysis buffer for 20 min. After removal of cellular debris by centrifugation (10 min at 9000 rpm at 4 °C), the supernatants were used to detect caspase-3 proteolytic activity. Samples were incubated with 200 μM caspase-3 substrate Ac-DEVD-pNA at 37 °C for 4 h and then analyzed at 405 nm in a microtiter plate reader. The protein levels in the lysates were measured with BCA protein assay kit (Pierce Biotechnology, Rockford, IL). NT and SR48692 effects on OGD-induced changes in caspsase-3 activity were evaluated by following the same procedure as described for endogenous glutamate experiments.

MAP2 Immunoreactivity

The neuronal damage was also performed by neurons immunochemical numeration through MAP2 immunocytochemistry. On DIV 8, cells were

rinsed in 0.1 M PBS and then fixed using 4% paraformaldehyde in Sorensen's buffer 0.1 M, pH 7.4, for 20 min. After rinses in PBS (3 times for 5 min each), the cells were incubated overnight at 4 °C with the primary antibody rabbit anti-MAP2. Anti-MAP2 antibody was diluted 1:1000 in PBS containing 0.3% Triton X-100 (v/v). The cells were then washed 3 times with PBS and incubated with rhodamine-conjugated antirabbit antibody (Chemicon, Temecula, CA) diluted 1:100 in PBS containing 0.3% Triton X-100 for 60 min at room temperature. After 3 washes in PBS, the cells were mounted in glycerol and PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope. Investigation of MAP2 aggregations in dendrites was performed with the ×100 magnification objective and on 30 randomly chosen fields in each coverslip. Subsequently, the number of aggregations was counted and referred to 100 μm of dendrite length (Image-Pro Plus 4.1; Immagini e Computer, Milan, Italy) (Pirondi et al. 2005). NT and SR48692 effects on OGD-induced increase of MAP2 immunoreactivity were evaluated by following the same procedure as described for endogenous glutamate experiments.

Materials

The culture dishes were purchased from Nunc A/S (Roskilde, Denmark). Neurobasal medium and B27 were obtained from Gibco (Grand Island, NY). Poly-L-lysine, trypsin, cytosine arabinoside, gentamycine sulfate, glutamine, L-glutamic acid, MTT, and Hoechst 33258 were obtained from Sigma Chemical Co., St Louis, Missouri. Anti-MAP2 antibody and rhodamine-conjugated antirabbit antibody were purchased from Chemicon. NT from Peninsula Laboratories Europe Ltd (Merseyside, UK) was dissolved in Krebs solution just before testing and used only once. SR48692 (2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl) carboxylamino]tri-cyclo(3.3.1.1.3.7)-decan-2-carboxylic acid) (Sanofi-Aventis, Montpellier, France) was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was < 0.001%; when required, the DMSO vehicle was added alone or together with NT, and no changes in glutamate efflux, LDH levels, mitochondrial enzymes activity, chromatin condensation, caspase-3 activity, annexin V staining, and MAP2 immunoreactivity were observed.

Statistical Analysis

Results are expressed throughout as means ± standard error of mean. The statistical analysis was carried out by analysis of variance followed by the Newman–Keuls test for multiple comparisons. $P < 0.05$ was the accepted level of significance.

Results

Endogenous Extracellular Glutamate Levels

Modulation of Endogenous Extracellular Glutamate Levels in Cortical Cell Cultures Exposed to OGD

The mean basal extracellular glutamate levels measured in the first 2 samples collected from cell cultures were 0.384 ± 0.021 μM ($n = 35$). As shown in Figure 1, endogenous extracellular glutamate levels, measured 24 h after OGD, were significantly increased (437 ± 35% of the respective basal value, $n = 15$) compared with control group not exposed to OGD (293 ± 21% of the respective basal value, $n = 10$).

Effects of the Exposure to NT and NT Receptor Antagonist SR48692 Alone or in Combination

The addition of NT (100 nM) to the cell cultures exposed to OGD was associated with a supplementary enhancement of the OGD-induced increase of endogenous extracellular glutamate levels (540 ± 47% of the respective basal value, $n = 14$). The exposure of cells to the NT antagonist SR48692 (100 nM) prevented the effect of the peptide on the OGD-induced increase of glutamate efflux (270 ± 28% of the respective basal value, $n = 14$). Interestingly, SR48692 100 nM by itself reduced the OGD-induced increase of endogenous extracellular glutamate levels (332 ± 37% of the respective basal value, $n = 14$) (Fig. 1).

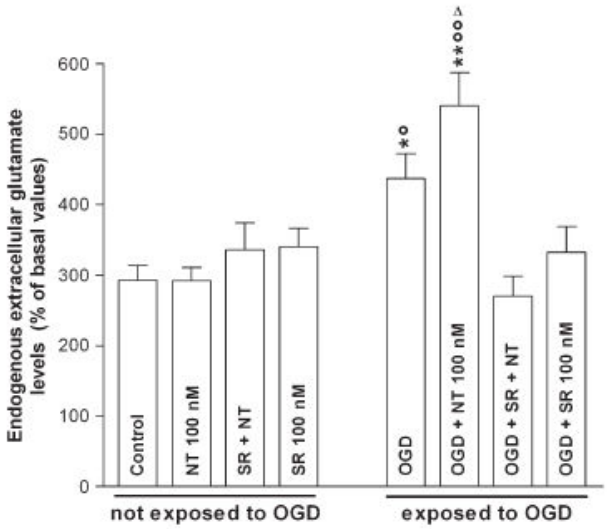

Figure 1. Effects of NT, SR48692 (SR), and NT + SR on endogenous extracellular glutamate levels in cortical cell cultures not exposed and exposed to OGD. Two samples, collected every 50 min, have been used to assess basal glutamate levels. A third fraction was collected 24 h later. The effects of the treatments on the endogenous extracellular glutamate levels in the third fraction are reported and expressed as percent changes of basal values, as calculated by the means of the 2 fractions collected prior to treatment. $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{\circ}P < 0.05$ and $^{\circ\circ}P < 0.01$ significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}P < 0.05$ significantly different from OGD according to analysis of variance followed by the Newman–Keuls test for multiple comparisons.

As shown in Figure1, when NT and SR48692 were added, alone or in combination, to cell cultures not exposed to OGD, they did not significantly influence the endogenous extracellular glutamate levels (292 ± 19%, $n = 15$; 340 ± 26%, $n = 10$; 336 ± 38%, $n = 10$, respectively) as compared with the control cell cultures.

Neuronal Death

LDH Release

The LDH level corresponding to complete neuronal death was determined by assaying sister cultures exposed to 1 mM glutamate for 24 h. The LDH values were expressed as percentage of the value found with complete neuronal death (100%).

The LDH release/absorbance, measured in cortical cell cultures not exposed to OGD, was 0.722 ± 0.04 ($n = 28$) and corresponds to 37 ± 2.8% of complete neuronal death. The exposure of cortical cell cultures to OGD induced a significant increase of LDH release (66 ± 2.6% of complete neuronal death, $n = 35$) with respect to control cell cultures not exposed to OGD (Fig. 2).

Effects of the Exposure to NT and NT Receptor Antagonist SR48692 Alone or in Combination

The addition of NT (100 nM) to the cultures further enhanced the OGD-induced increase of LDH release (82 ± 2.8% of

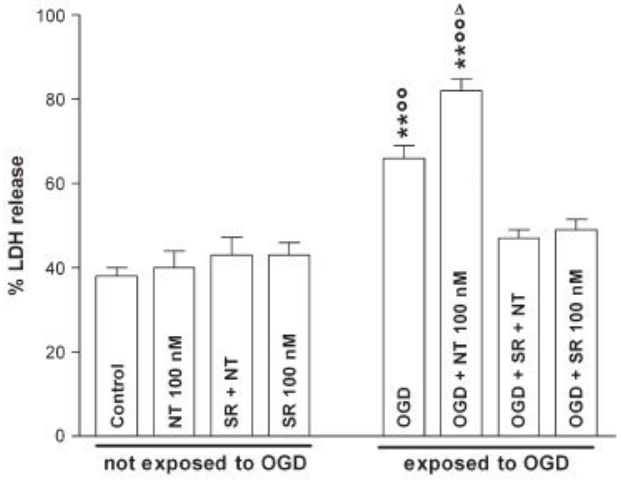

Figure 2. Effects of NT, SR48692 (SR), and NT + SR on LDH release in cortical cell cultures not exposed and exposed to OGD. The LDH values are expressed as percentage of the value found with complete neuronal death (100%), determined by assaying sister cultures exposed to 1 mM glutamate for 24 h. $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{\circ\circ}P < 0.01$ significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}P < 0.05$ significantly different from OGD according to analysis of variance followed by the Newman–Keuls test for multiple comparisons.

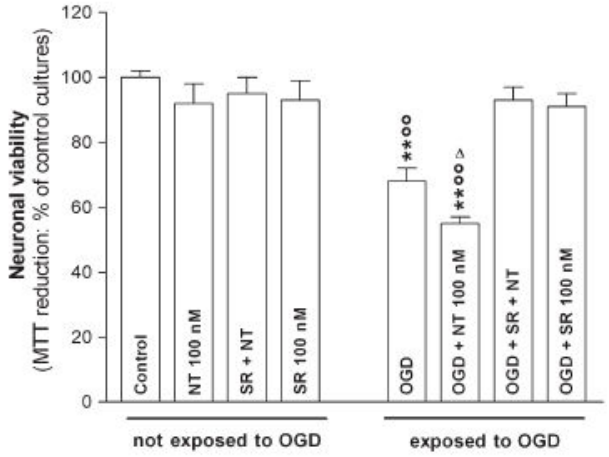

Figure 3. Effects of NT, SR48692 (SR), and NT + SR on neuronal viability (expressed as MTT reduction) in cortical cell cultures not exposed and exposed to OGD. $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{\circ\circ}P < 0.01$ significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}P < 0.05$ significantly different from OGD according to analysis of variance followed by the Newman–Keuls test for multiple comparisons.

complete neuronal death, $n = 23$). The selective NT receptor antagonist SR48692 (100 nM) prevented not only the effect of the peptide but also the OGD-induced increase of LDH release (47 ± 2.5% of complete neuronal death, $n = 25$, and 49 ± 2.5% of complete neuronal death, $n = 28$, respectively; Fig. 2).

Any effects were observed after the addition of NT and SR48692, alone or in combination, to the cultures not exposed to OGD (40 ± 4% of complete neuronal death, $n = 22$; 43 ± 3% of complete neuronal death, $n = 23$; 43 ± 4% of complete neuronal death, $n = 21$, respectively; Fig. 2).

MTT Assay

The MTT absorbance values, measured in cortical cell cultures not exposed to OGD, were 0.125 ± 0.02, $n = 20$.

Exposure of cortical cell cultures to OGD induced an impairment of oxidative ability of mitochondria, as indicated by MTT reduction, as measured after the insult (68 ± 4% of control value, $n = 20$; Fig. 3).

Effects of the Exposure to NT and NT Receptor Antagonist SR48692 Alone or in Combination

The addition of NT (100 nM) to the cultures further decreased the OGD-induced MTT reduction (55 ± 2% of control value, $n = 20$). SR48692 (100 nM) prevented not only the effect of the peptide but also the OGD-induced MTT reduction (93 ± 4% of control value, $n = 18$; 91 ± 4% of control value, $n = 18$, respectively; Fig. 3).

Any effects were observed after the addition of NT and SR48692, alone or in combination, to the cultures not exposed to OGD (92 ± 6% of control value, $n = 10$; 93 ± 6% of control value, $n = 12$; 95 ± 5% of control value, $n = 12$, respectively; Fig. 3).

Nuclear Staining with Hoechst 33258

The specific DNA stain Hoechst 33258 was used to assess changes in chromatin and nuclear structure. As reported in a representative fluorescence photomicrograph (Fig. 4*A*) nuclei of viable cells (control group, not exposed to OGD) exhibited a large and diffuse chromatin staining. In contrast nuclei of cortical cells exposed to OGD showed a variety of abnormal morphologies including highly condensed and fragmented chromatin (Fig. 4*B*). All nuclei with morphological abnormalities were considered "condensed." In OGD-exposed cell cultures, the percentage of cells with altered nuclear morphology (fragmented nuclei) was significantly higher than that observed in control cell cultures not exposed to OGD (55 ± 3.8%, $n = 18$, and 32 ± 2.3%, $n = 15$, respectively; Fig. 5).

Effects of the Exposure to NT and NT Receptor Antagonist SR48692 Alone and in Combination

The addition of NT (100 nM) to the cultures further enhanced the OGD-induced increase in nuclear fragmentation (Fig. 4*C*) and in the percentage of fragmented nuclei (71 ± 3%, $n = 9$; Fig. 5). In the presence of the selective NT receptor antagonist SR48692 (100 nM), not only the NT-induced effect was counteracted (Fig. 4*D*) but also the OGD-induced increase of the percentage of fragmented nuclei was prevented (41 ± 1.4%, $n = 9$, and 43 ± 1.5%, $n = 8$, respectively; Fig. 5).

Any effects were observed after the addition of NT and SR48692, alone or in combination, to the cultures not exposed to OGD (35 ± 4%, $n = 8$; 33 ± 3%, $n = 8$; 34 ± 4%, $n = 8$ respectively; Fig. 5).

Annexin V Staining

AN/PI double staining was used to demonstrate that translocation of phosphatidyl serine from the inner to the outer leaflet, a characteristic feature of apoptotic death, occurs after OGD in cortical cell cultures. The cells stained by annexin V but not by propidium iodide [AN(+)/PI(−)] are those that undergo apoptotic cell death. In normoxic conditions, the cultures showed that 4.5 ± 0.5% ($n = 9$) of the total cells counted were AN(+)/PI(−), whereas in the OGD-exposed cultures the number of AN(+)/PI(−) immunoreactive cells increased to 9 ± 0.9% ($n = 9$) (Fig. 6).

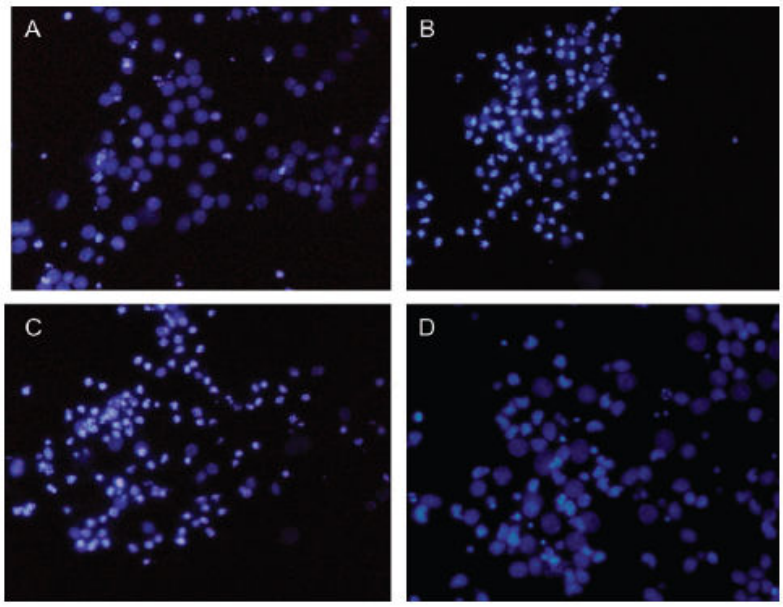

Figure 4. Representative fluorescence photomicrographs of cells with nuclear fragmentation in cortical cell cultures not exposed (panel *A*) and exposed to OGD (panel *B*), to OGD + NT (panel *C*), and to OGD + NT + SR48692 (panel *D*). The neurons were stained with Hoechst 33258 and observed in sampled fields under fluorescent microscope (magnification ×20).

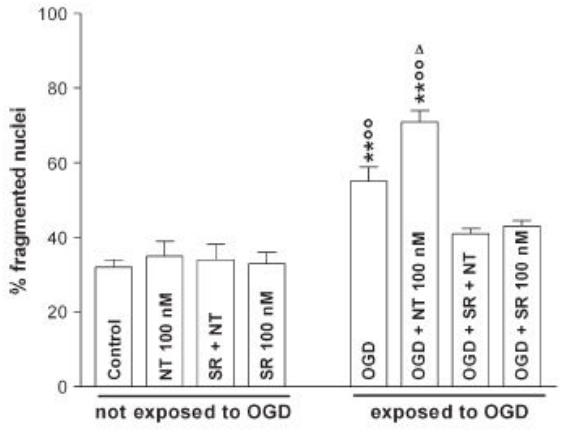

Figure 5. Effects of NT, SR48692 (SR), and NT + SR on the percentage of fragmented nuclei in cortical cell cultures not exposed and exposed to OGD. $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{oo}P < 0.01$ significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}P < 0.05$ significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

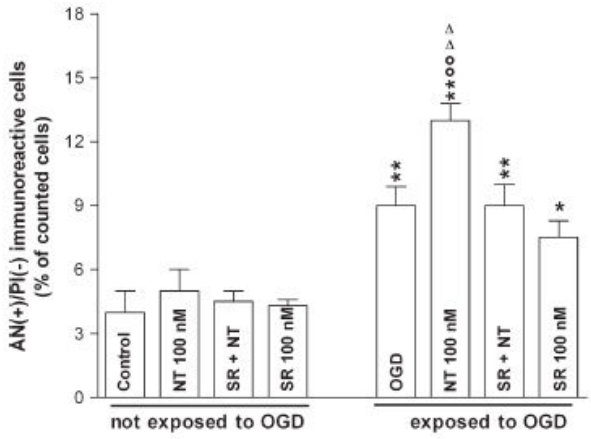

Figure 6. Effects of NT, SR48692 (SR), and NT + SR on the percentage of AN(+)/PI(−) immunoreactive cells in cortical cell cultures not exposed and exposed to OGD. $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{oo}P < 0.01$ significantly different from OGD + SR and OGD+ SR + NT; $^{\Delta\Delta}P < 0.01$ significantly different from OGD according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Effects of the Exposure to NT and NT Receptor Antagonist SR48692 Alone and in Combination

The addition of NT (100 nM) to the cultures further enhanced the OGD-induced increase in the percentage of AN(+)/PI(−) immunoreactive cells (13 ± 0.8%, $n = 9$; Fig. 6). In the presence of the selective NT receptor antagonist SR48692 (100 nM), the NT-induced effect was counteracted (9 ± 1%, $n = 8$; Fig. 6).

Any effects were observed after the addition of NT and SR48692, alone or in combination, to the cultures not exposed to OGD (5 ± 1%, $n = 8$; 4 ± 0.3%, $n = 8$; 4.5 ± 0.5%, $n = 8$, respectively; Fig. 6).

Caspase-3 Activity

In cortical cell cultures not exposed to OGD, the caspase-3 activity was 6.9 ± 0.6 (pNA pmol/min/mg protein; $n = 10$). Exposure of cortical cell cultures to OGD induced an increase of caspase-3 activity (135 ± 10% of control value, $n = 10$; Fig. 7).

Effects of the Exposure to NT and NT Receptor Antagonist SR48692 Alone or in Combination

The addition of NT (100 nM) to the cultures further enhanced the OGD-induced increase of caspase-3 activity (206 ± 18% of

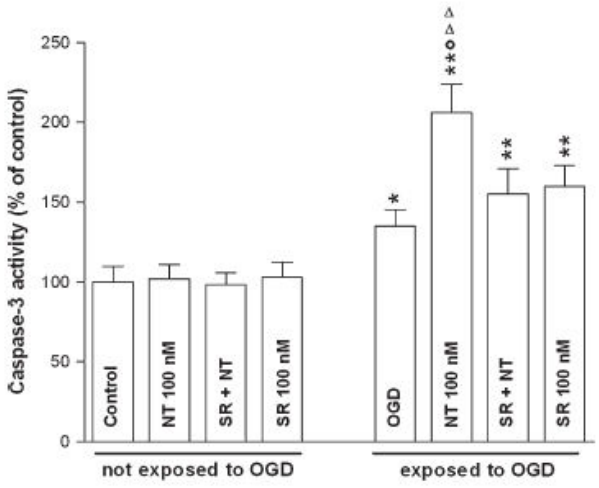

Figure 7. Effects of NT, SR48692 (SR), and NT + SR on the caspase-3 activity expressed as percentage of control values in cortical cell cultures not exposed and exposed to OGD. $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{\circ}P < 0.05$ significantly different from OGD + SR and OGD+ SR + NT; $^{\Delta\Delta}P < 0.01$ significantly different from OGD according to analysis of variance followed by the Newman–Keuls test for multiple comparisons.

control value, $n = 10$; Fig. 7). In the presence of SR48692 (100 nM), the NT-induced increase of caspase-3 activity was counteracted (155 ± 16% of control value, $n = 10$; Fig. 7).

Any effects were observed after the addition of NT and SR48692, alone or in combination, to the cultures not exposed to OGD (102 ± 9% of control value, $n = 10$; 103 ± 9.5% of control value, $n = 12$; 98 ± 8% of control value, $n = 10$, respectively; Fig. 7).

MAP2 Immunoreactivity

As MAP2 is described as an early indicator of ischemia-induced neurodegeneration, the cortical cell cultures were MAP2 immunostained. In normoxic conditions, the cultures showed a high number of healthy neurons and a neuronal network formed by highly arborized dendritic trees and an homogeneously diffused MAP2 immunoreactivity of the cell bodies and dendrites (see Fig. 8*A,A'*). On the contrary, a loss of MAP2 immunoreactivity was observed after OGD in particular in the dendrites, which often appeared to be truncated, as showed by the different distribution of MAP2 immunoreactivity along the dendrites (Fig. 8*D,D'*).

To quantify the effect of OGD on cell morphology, the number of MAP2 aggregations in dendrites was counted and referred to 100 μm of dendrite length sections. As shown in

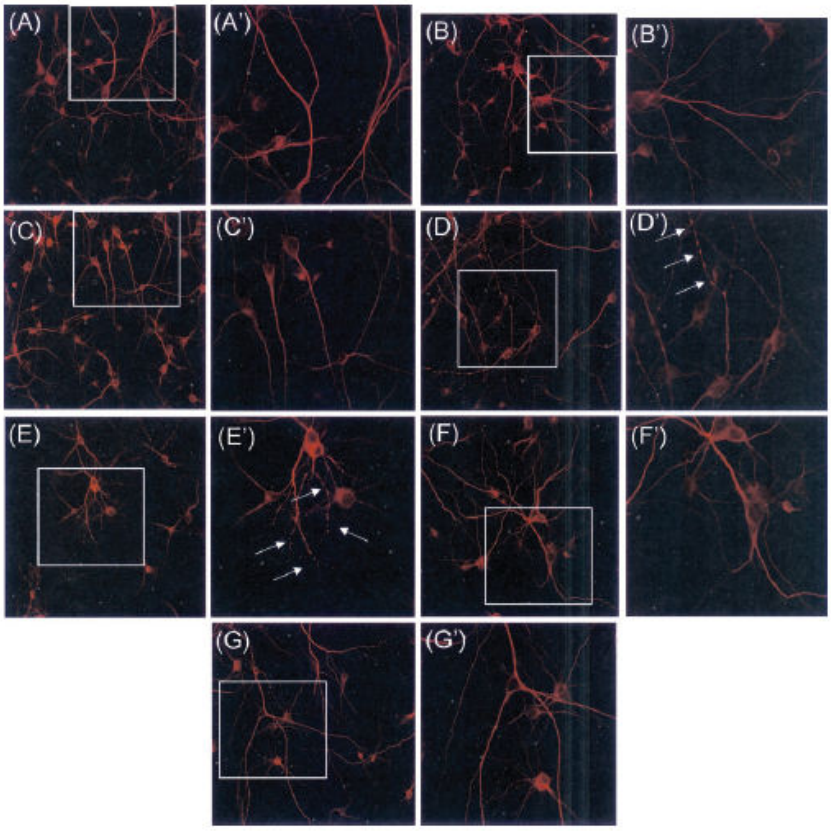

Figure 8. Representative fluorescence photomicrographs of MAP2 immunoreactivity in cortical cell cultures not exposed (control: *A, A'*) and exposed to OGD (*D, D'*). Effects of NT 100 nM (*B, B'*) and SR48692 100 nM (*C, C'*) in cortical cell cultures not exposed to OGD. Effects of NT 100 nM (*E, E'*), SR48692 100 nM (*F, F'*), and NT 100 nM + SR48692 100 nM (*G, G'*) in cortical cell cultures exposed to OGD. Surviving neurons were stained with anti-MAP2 antibody and observed in sampled fields under fluorescent microscope 24 h after OGD (magnification ×20: *A, B, C, D, E, F, G*; magnification ×40: *A', B', C', D', E', F', G'*).

Figure 9, in control cultures not exposed to OGD, MAP2 aggregations per length unit were 1.24 ± 0.98 aggregation per 100 μm (n = 10), whereas cultures exposed to OGD showed a marked increase (12.30 ± 1.10 aggregation/100 μm, n = 10).

Effects of the Exposure to NT and NT Antagonist SR48692 Alone and in Combination

When NT (100 nM) was added to the OGD-exposed cultures, the fragmented distribution of MAP2 immunostaining along the dendrites became more noticeable (Fig. 8*E,E'*), and a further significant increase in MAP2 aggregations was observed (17.55 ± 1.21 aggregation per 100 μm; Fig. 9). The addition of SR48692 (100 nM) not only counteracted the NT-induced effect but also the OGD-induced decrease of dendrite outgrowth (Fig. 8*F,F'*, respectively) and OGD-induced increase of MAP2 aggregations (1.87 ± 0.69 aggregation per 100 μm, n = 11; 2.12 ± 0.95 aggregation per 100 μm, n = 11, respectively; Fig. 9).

The addition of NT and SR48692, alone or in combination, to the cultures not exposed to OGD did not modify the dendrite outgrowth (Fig. 8*B,B',C,C'*, respectively) and the number of MAP2 aggregations (1.30 ± 0.75 aggregation per 100 μm, n = 8; 0.53 ± 0.09 aggregation per 100 μm, n = 8; 1.39 ± 0.85 aggregation per 100 μm, n = 8, respectively; Fig. 9).

Discussion

The OGD is an in vitro model of ischemia (Strasser and Fischer 1995) that has been used in this study to define the role of NT in OGD-induced neuronal death in cortical cell cultures and also to give a pathological correlate to the ability of NT to enhance NMDA-induced glutamate release (Antonelli et al. 2004). Furthermore, glucose insufficiency reduces neuronal viability and increases caspase-3 activity linked to increased LDH levels (Ioudina et al. 2004). Both necrotic and apoptotic neuronal death have been described following cerebral ischemia.

The use of diverse methods for measuring cell death should help to define causal relationship between the mechanisms that regulate apoptosis and the cell death event itself. Biochemical methods such as LDH release and MTT enzyme activity, used to quantify the cell death, analyze different cellular events. Indeed, LDH release serves as an indicator of loss of cell membrane integrity and thus cell death. The MTT assay provides an indirect measurement of cell growth/cell death through measurement of the ability of mitochondria to convert MTT to formazon. This assay measures mitochondrial function and not cell death per se. The measurement of loss of mitochondrial function will be a late indicator of apoptotic cell death because mitochondria often remain intact until late in apoptosis. These biochemical methods present several drawbacks; indeed, both LDH release and MTT assay do not discriminate between apoptosis and necrosis. Thus, different methods are required to confirm apoptosis (Loo and Rillema 1998). Apoptosis is characterized by nuclear changes, that is, aggregation of chromatin at the nuclear membrane, membrane blebbing without loss of integrity, and chromatin fragmentation (Simm et al. 1997). For this reason, the specific DNA stain, Hoechst 33258, was used to assess changes in chromatin and nuclear structure following OGD. Furthermore, caspase-3 activity and annexin V assay were used to confirm the presence of the apoptotic component in OGD-induced neuronal cell death. Annexin V assay is based on the observation that during induction of apoptosis, phosphatidyl serine (PS) is translocated from the inner leaflet to the outer leaflet of the plasma membrane (Huerta et al. 2007). Under the OGD condition, cultured neurons exhibited annexin V labeling on the periphery of their soma and dendrites while PI was excluded. These data confirm that PS is externalized, whereas the plasma membrane remains intact, strongly supporting the occurrence of apoptosis. Finally, the MAP2 family of proteins is an abundant group of cytoskeletal components that are predominantly expressed in neurons and have been proposed to play important roles in the outgrowth of neuronal processes, synaptic plasticity, and neuronal cell death. MAP2 is depleted after in vivo and in vitro ischemia (Li et al. 2000; Kuhn et al. 2005), and for this reason, MAP2 immunoreactivity may be considered an early indicator of ischemia-induced neurodegeneration. (Buddle et al. 2003). Thus, by measuring extracellular glutamate levels, LDH levels, mitochondrial dehydrogenase activity, apoptotic nerve cell death with Hoechst 33258, annexin V and caspase-3 activity, and MAP2 immunoreactivity, we provide biochemical and morphological evidence that NT, via the activation of NTR1, is involved in causing neuronal death upon OGD in cortical cultures. In fact, the addition to the incubation medium of exogenous NT at nanomolar concentrations during OGD produces a further and significant enhancement of the LDH levels and a further significant decrease of mitochondrial functionality with respect to the LDH and MTT levels observed during OGD alone. In line with the above finding, it has been shown that the NTR1 antagonist SR48692 can increase cell membrane integrity and cell viability.

The possible role of the NTR1 in degeneration induced by OGD is further supported by the results obtained in the experiments where the apoptotic cell death is analyzed. In fact, these data indicate that the peptide on its own enhances, whereas the NT receptor antagonist blocks the OGD-induced increase in the number of fragmented nuclei and AN(+)/PI(–) immunoreactive cells as well as caspase-3 activity in the cortical cultures. In addition, the outgrowth of cortical dendrites, already altered after OGD (Matesic and Lin 1994;

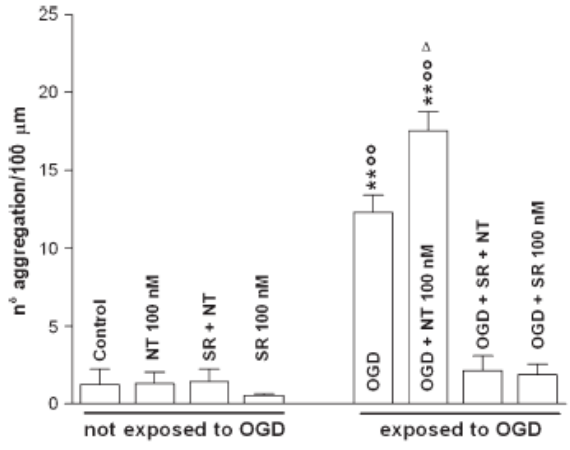

Figure 9. Effects of NT, SR48692 (SR), and NT + SR on the number of MAP2 aggregations per 100 μm length in cortical cell cultures not exposed and exposed to OGD. $^{**}P < 0.01$ significantly different from control as well as NT, SR, and NT + SR (not exposed to OGD); $^{\circ\circ}P < 0.01$ significantly different from OGD + SR and OGD + SR + NT; $^{\Delta}P < 0.05$ significantly different from OGD according to analysis of variance followed by the Newman–Keuls test for multiple comparisons.

Vanicky et al. 1995), are further impaired in the presence of NT, as demonstrated by the MAP2 immunoreactivity experiments. The injury to the neuronal dendrites, which may be observed before neuronal death, is characterized by a reduction of outgrowth and branching of dendrites as well as by an increase of the number of MAP2 aggregations. This alteration in dendrite morphology is an early consequence of excessive glutamate release occurring during ischemia (Esquenazi et al. 2002). The involvement of NTR1 in the OGD-induced reduction of neuronal population and dendritic outgrowth is confirmed by the treatment with SR48692, which antagonizes the OGD-induced neurodegeneration.

Thus, taken all together these biochemical and morphological results lead to hypothesize that under normothermic conditions NTR1 activation may contribute to apoptotic nerve cell death induced by OGD with reduction of neuronal survival (see Ioudina et al. 2004).

Among the myriad of biochemical events triggered by cerebral ischemia, increase in Ca^{2+} influx, formation of free radicals, loss of adenosine triphosphate, etc., there is also an increase in extracellular concentration of neurotransmitters, especially of glutamate. In this context, it is well known that substantial elevation in extracellular glutamate levels and, consequently, the excessive stimulation of excitatory amino-acid receptors is implicated in the neuronal cell death occurring under these degenerative processes (Choi et al. 1988; Arundine and Tymianski 2004; Young et al. 2004; Zipfel et al. 2000). Thus, in view of the results obtained in the present study, it could be suggested that one of the possible mechanisms that leads to NT-mediated apoptotic nerve cell death and NT-mediated reduction of dendritic outgrowth and branching could involve the ability of the peptide to modulate the glutamatergic transmission.

Such a hypothetical mechanism seems to be confirmed by the results indicating that during the OGD, the presence of NT in the medium induces a significant amplification in the rise of extracellular glutamate levels compared with that observed during OGD alone. This view is strengthened by the result indicating that the NT receptor antagonist SR48692 counteracts the OGD-induced rise of the extracellular levels of glutamate reducing the endogenous levels of glutamate to the values observed in control neuronal cells. Interestingly, SR48692 is able to counteract some biochemical and morphological OGD-induced alterations also in the absence of exogenous NT. These findings can be explained by release of endogenous NT from neuronal and/or glial cells under OGD increasing NTR1 tone that can be blocked by the NTR1 receptor antagonist. In line with this explanation, a rise of NT immunoreactivity in certain brain regions has been found in the rat middle cerebral artery occlusion model of stroke (Allen et al. 1995). However, SR48692 by itself did not reduce the caspase-3 activation and the number of AN(+)/PI(−) immunoreactive cells induced by OGD. At the present, this discrepancy could be attributed to the different methodological approaches used to determine neurodegeneration and remain to be clarified in further studies.

It is worth noting that previous results performed in mesencephalic cell cultures containing dopaminergic neurons that express functional NT receptors demonstrate that the neurotoxic effects of glutamate are exacerbated by NT when the peptide is applied in combination with exogenous glutamate (Antonelli et al. 2002). NTR1 is coupled to phospholipase C and will therefore upon activation increase protein kinase C activity (see Kinkead and Nemeroff 2004), and it has previously been demonstrated that NT receptors enhance NMDA-induced excitotoxicity in cortical cultures via an activation of protein kinase C (Antonelli et al. 2004). Therefore, it may be speculated that under the present pathological conditions, the peptide both by enhancing glutamate release and the NT-NMDA receptor interactions could amplify the glutamate signal transduction contributing to neuronal injury after both OGD and probably after ischemia. Thus, it is known that protein kinase C when activated can increase the opening rate of the NMDA channels and recruit new NMDA channels to the surface membrane (see Lan et al. 2001). This amplification of NMDA signaling by NT could explain the increase of MAP2 aggregations occurring after OGD. In fact, following NMDA receptor activation, the calcium influx, through the receptor ion channel complex, and the release of calcium from the mitochondria, through the activation of the $2Na^{+}/Ca^{++}$ exchanger, trigger MAP2 degradation (Buddle et al. 2003).

Hypothermia affects a wide variety of processes involved in the cerebral ischemia, and in both models of global and focal cerebral ischemia, it has been shown that hypothermia could be neuroprotective (Corbett and Thornhill 2000; Gordon 2001; Krieger and Yenari 2004). Thus, an alternative to the classical pharmacological approach to obtain neuroprotection could be the administration of a compound that induces hypothermia. In this context, it has been suggested that the hypothermic effect of NT could be the mechanism involved in the reduction of the CA1 damage in hippocampus after global ischemia in mongolian gerbils (Babcock et al. 1993). In addition, NT and NT analogues have been reported to produce hypothermia in rodents (Bissette et al. 1976; Dubuc et al. 1992), and the neuroprotective effect of the NT analogue H-Lys-psi (CH_2NH)Lys-Pro-Tyr-Ile-Leu-OH (JMV-449) has been demonstrated in a mouse model of permanent distal middle cerebral artery occlusion (Torup et al. 2003). Such a neuroprotection is likely to be mediated via the systemic hypothermia as no neuroprotective effect was seen if the JMV-449–treated mice were kept normothermic (Torup et al. 2003). However, it must be noted as shown in the present study that the NTR1 antagonist SR48692 in vitro counteracts the local neurodegeneration-enhancing effects of NT, whereas the hypothermic effects of NT in vivo are not counteracted by this NTR1 antagonist (Gully et al. 1995, 1997). Thus, NTR1 antagonist such as SR48692 may offer a new treatment possibility of ischemia.

In conclusion, taken together the present results, obtained in the OGD model in cortical cultures representing an in vitro model of cortical ischemia, suggest that cortical NT receptor activation may contribute to neuronal injury after ischemia. The NT receptor antagonists could provide a new tool to explore the clinical possibilities and thus to move from chemical compound to effective drug.

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Address correspondence to Dr Luca Ferraro, Department of Clinical and Experimental Medicine, Pharmacology Section, University of Ferrara, Via Fossato di Mortara 17-19, 44100 Ferrara, Italy. Email: frl@unife.it.

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BRAIN RESEARCH REVIEWS

Review

Neurotensin receptors as modulators of glutamatergic transmission

Luca Ferraro[a], *Maria Cristina Tomasini*[a], *Roberta Mazza*[a], *Kjell Fuxe*[b], *Jacqueline Fournier*[c], *Sergio Tanganelli*[a,*], *Tiziana Antonelli*[a]

[a]*Department of Clinical and Experimental Medicine, Section of Pharmacology, University of Ferrara, Via Fossato di Mortara 17-19, 44100 Ferrara, Italy*
[b]*Department of Neuroscience, Division of Cellular and Molecular Neurochemistry, Karolinska Institutet, Stockholm, Sweden*
[c]*Sanofi-Aventis, Neurology Section, 195 route d'Espagne, 31036 Toulouse Cedex, France*

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ABSTRACT

Functional studies have provided evidence supporting the concept that the tridecapeptide neurotensin (NT) acts in the central nervous system as a classical neurotransmitter and/or as an important modulator of neuronal signalling. The role of NT in the regulation of the striatal aminoacidergic transmission, mainly by antagonising D2 receptor function, will be analysed. In addition, in different rat brain regions, including the basal ganglia, the contribution of NT receptors in modulating and reinforcing glutamate signalling will be shown including the involvement of interactions between NT and NMDA receptors. Since the enhancement of glutamate transmission and in particular the excessive activation of NMDA receptors, has been postulated to be an important factor in the induction of glutamate-mediated neuronal damage, the involvement of NT in the glutamate-induced neurodegenerative effects will be discussed. Moving from these observations and in order to further investigate this issue, results from preliminary behavioural, functional and biochemical experiments will be presented on the putative neuroprotective effect obtained by the blockade of NT receptor 1 (NTS1) via the systemic administration of the selective NTS1 antagonist SR48692 in an *in vivo* animal model of Parkinson's disease [unilateral nigral 6-hydroxydopamine (6-OHDA) induced lesion of the nigrostriatal pathway].

Contents

* *Corresponding author*. Fax: +39 0532 455205.
E-mail address: tgs@unife.it (S. Tanganelli).
Abbreviations: DA, dopamine; NT, Neurotensin; NTS1, NT receptor 1; 6-OHDA, 6-hydroxydopamine

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1. Introduction

Neurotensin (NT) is a tridecapeptide, widely distributed throughout the central nervous system as well as different peripheral tissues of mammals, including humans. In the brain, NT is particularly expressed within the nigrostriatal and mesolimbic dopamine (DA) system (Jennes et al., 1982; Leeman and Carraway, 1982) and most of its effects are mediated via the activation of two different G protein-coupled receptor subtypes: named NTS1 and NTS2 (Vincent et al., 1999; Pelaprat, 2006). The close co-distribution between NT and DA systems together with the functional evidence that NT modulates dopaminergic signalling both in limbic and in striatal brain regions have suggested that NT may be implicated in the pathophysiology of various central nervous system disorders including schizophrenia and Parkinson's disease (Nemeroff, 1980; McMahon et al., 2002; Caceda et al., 2006; Kinkead and Nemeroff, 2006; St-Gelais et al., 2006). Within the basal ganglia NT is closely associated with the nigrostriatal DA and striatopallidal GABA neurons. Its high affinity receptor (NTS1) is widely expressed (mRNA) on dopaminergic neurons into the substantia nigra. In the striatum, its expression seems to be prevalently associated to the dopaminergic terminals from the nigral neurons and glutamatergic terminals from cortical inputs (Nicot et al., 1994; Alexander and Leeman, 1998). The possible location of NTS1 on axon terminals suggests that NT may modulate dopaminergic neuronal transmission by acting presynaptically on receptors located on either dopaminergic or non-dopaminergic afferent terminals. The presence of NTS1 on presynaptic DA terminals in the caudate was confirmed by the findings obtained using 6-hydroxydopamine (6-OHDA) lesioned rats (Herve et al., 1986; Dilts and Kalivas, 1989; Schotte and Leysen, 1989; Masuo et al., 1990; Cadet et al., 1991). Moreover, experiments performed by lesioning intrinsic striatal neurons or cortico-striatal projections, suggest that NTS1 are not exclusively located on DA terminals (Goedert et al., 1984; Masuo et al., 1990).

There is evidence that in the globus pallidus, NT receptors (NTS1 and NTS2) exist in different neurons and are located both presynaptically and postsynaptically (Fassio et al., 2000; Sarret et al., 2003) thus modulating, mainly via NTS1 receptors, both pallidal glutamatergic and GABAergic transmission (Chen et al., 2004; 2006). Since Binder et al. (2001) suggested that in the striatum "30% of NTRs may be located on DA terminals, 50% on intrinsic neurons and 20% on cortico-striatal projections", and according to the heterogeneous distribution of NTRs in the globus pallidus, both receptors could also be expressed in the caudate, NTS1 modulating the dopamine and glutamate release from nigral and cortical inputs.

The aim of the present review is to summarize in the first part the neurochemical evidence, in particular from our laboratory, which emphasizes the role of NT in the modulation of the striatal aminoacidergic and dopaminergic signalling, mainly by antagonising D2 receptor function. In the second part the contribution of NTS1 receptors in modulating and reinforcing glutamate signalling is dealt with as well as the interactions between NTS1 and NMDA receptors in different rat brain regions, including the basal ganglia. Finally, in view of the existence of a significant enhancement by NT of the glutamatergic signalling that mainly involves NMDA receptors, the putative involvement of NT in neurodegenerative disorders is discussed. In this context, preliminary data on the protective role of NTS1 antagonist on nigrostriatal DA neurons in 6-OHDA-lesioned rats are presented.

2. Microdialysis evidence on the existence of a functional antagonistic NTS1 and D2 receptor interaction within the striatum

In previous papers by using mono and dual probe microdialysis techniques we provided evidence that NT regulates limbic and striatal dopaminergic function as well as striatopallidal GABAergic pathways mainly by antagonising D2 receptor function through a NTS1/D2 receptor–receptor interaction located at the pre- and post-synaptic level (Fuxe et al., 1992). We are now able to provide *in vivo* evidence indicating that NT modulation of glutamatergic cortico-striatal neurons in the striatum takes place also via an interaction with the dopamine D2 receptor. In this new set of experiments a microdialysis probe was implanted into the striatum of awake rats and as expected, 10-min pulse of high K^+ (50 mM) Ringer solution significantly increased extracellular glutamate levels (basal values: 0.292± 0.033 M; K^+ (50 mM) Ringer solution: 0.610±0.041; 209±7% of basal values). The perfusion with the dopamine D2 agonist quinpirole (10 μM) significantly reduced the K^+-evoked

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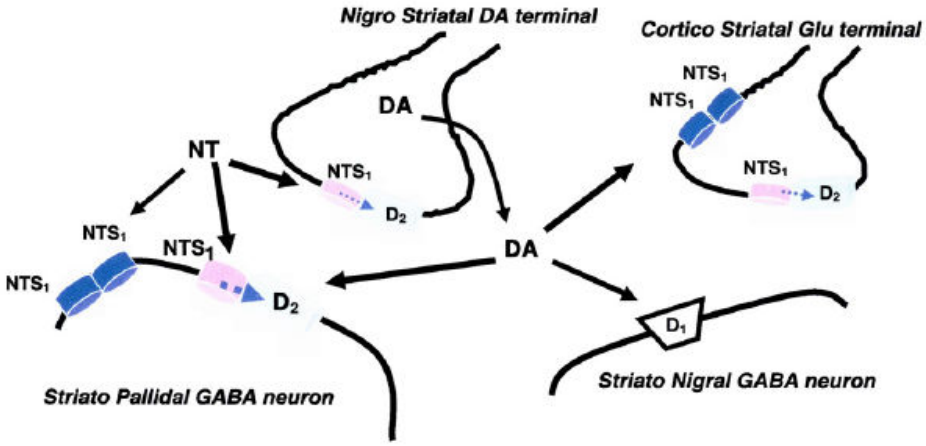

Fig. 1 – Schematic representation of the pre- and postjunctional neurotensin (NT) receptor (NTS1) and dopamine (DA) D2 receptor interactions in the striatum taking place in NTS1/D2 heteromers in balance with excitatory NT receptor homomers.

glutamate release (down to 163±9% of basal values). Interestingly, the addition into the perfusate medium of a low ineffective concentration (10 nM) of NT or its biologically active carboxy-terminal fragment NT(8–13), in combination with the D2 agonist (10 μM) counteracted the inhibitory effect of quinpirole on striatal K^+-evoked glutamate outflow (216±6% and 211±10% of basal values, respectively). These results outline, for the first time, the existence of a functional antagonistic presynaptic NTS1 receptor modulation of D2 receptor signalling at the terminal level of the glutamatergic cortico-striatal neurons. In summary, the present and the

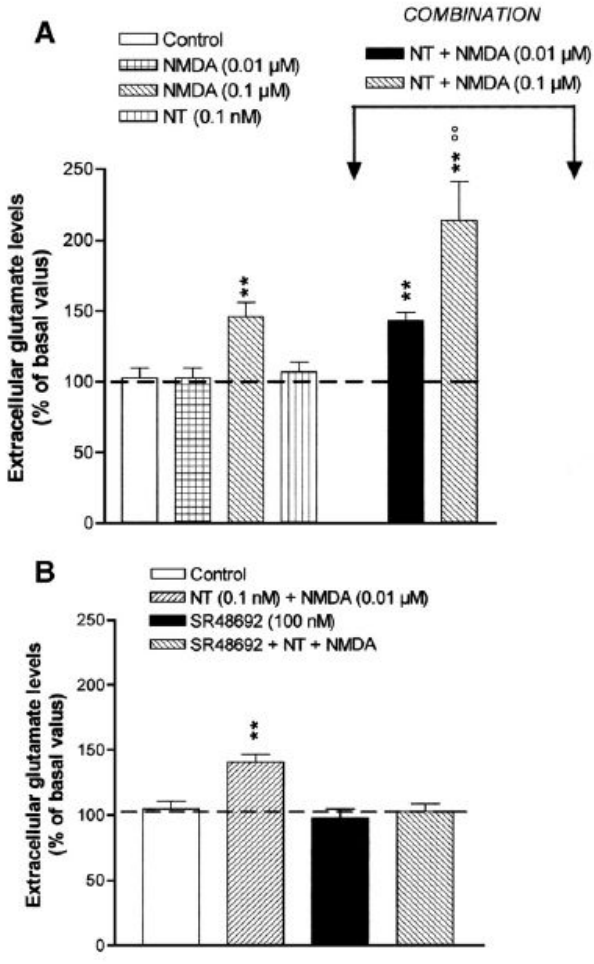

Fig. 2 – Panel A. Effects of treatments with NT (0.1 nM) and NMDA (0.01 and 0.1 μM), alone and in combination, on extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.099±0.003 μM), calculated from the mean of the first two fractions. NT was applied at the onset of the third fraction and maintained for 30 min, while NMDA was added to the Mg^{2+}-free Krebs–Ringer solution 10 min before the end of the third fraction. Each column represents the mean±SEM of 13 or 14 experiments for each group. $^{**}p<0.01$ significantly different from control, NMDA (0.01 μM) as well as NT (0.1 nM); $^{°°}p<0.01$ significantly different from all the other groups, according to the two-way ANOVA followed by the Newman–Keuls test for multiple comparisons (Antonelli et al., 2004). Panel B. Effects of SR48692 (100 nM) on the NT (0.1 nM)+NMDA (0.01 μM)-induced increase in extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.102± 0.009 μM), calculated from the mean of the first two fractions. NT was applied at the onset of the third fraction and maintained for 30 min, while SR48692 was added 20 min before the agonist. NMDA was added to the Mg^{2+}-free Krebs–Ringer solution 10 min before the end of the third fraction. Each column represents the mean±SEM of seven or eight experiments for each group. $^{**}p<0.01$ significantly different from the other groups, according to the two-way ANOVA followed by the Newman–Keuls test for multiple comparisons (Antonelli et al., 2004).

previous microdialysis studies (Fuxe et al., 1992; Tanganelli et al., 1994; Ferraro et al., 1995, 1997, 1998, 2001) suggest that within the striatum the antagonistic NTS1/D2 receptor–receptor interaction exist both at the presynaptic level of dopaminergic nigrostriatal neurons and glutamatergic cortico-striatal neurons as well as at the post-synaptic level on GABAergic striatopallidal neurons. Thus, from a functional point of view, it could be considered that the inhibitory modulation of NTS1 exerted on D2 receptor signalling which takes place via an antagonistic NTS1/D2 receptor–receptor interaction, may represent a major integrative mechanism that mediates the neurobiological effects of NT (Fig. 1), especially when the peptide is present in threshold concentrations within the striatum.

3. Neurotensin and glutamate-induced excitotoxicity

Glutamate is the major excitatory neurotransmitter in the central nervous system of vertebrates and several lines of evidence have led to formulate the hypothesis that abnormalities in glutamate transmission, particularly involving excessive NMDA receptor activation, may contribute to neurodegeneration associated with Parkinson's disease, Alzheimer's disease, ischemic events, hypoxia, and hypoglycemia (Choi et al., 1988). The mechanism(s) underlying the excitotoxicity induced by glutamate is, however, not completely understood, although there is ample evidence that it depends on the intensity of the glutamate insult (Cheung et al., 1998). Therefore, endogenous compounds able to modulate the glutamatergic signalling may interfere with the mechanisms involved in glutamate-induced cell death: by necrosis (intense glutamate insult) (Cheung et al., 1998; Zipfel et al., 2000) or by apoptosis (mild glutamate insult) (Bonfoco et al., 1995; Finiels et al., 1995; Cheung et al., 1998).

In the following paragraph, functional findings showing that NT modulates glutamatergic transmission in different brain regions including the basal ganglia will be summarized.

3.1. *Evidence for the existence of NT/NMDA receptor interactions on the modulation of striatal and cortical glutamate transmission*

It is well known that the substantial elevation in extracellular glutamate levels and consequently the excessive stimulation of glutamate receptors, especially NMDA receptors, can lead to excitotoxicity, which is part of the pathogenesis of neuronal cell death occurring during degenerative processes. In the next section of the manuscript, *in vitro* and *in vivo* evidences will be presented indicating that NT modulates striatal and cortical glutamate transmission and that NT and NMDA receptor interactions play a role in the regulation of endogenous cortical and striatal glutamate release.

3.1.1. *In vitro NT/NMDA receptor interactions on extracellular glutamate levels from primary cultures of rat cortical neurons*

As summarized in Fig. 2, *in vitro* experiments, performed on primary cell cultures of rat cortical neurons (Antonelli et al., 2004), demonstrated that when ineffective concentrations of both NT (0.1 nM) and NMDA (0.01 μM), were simultaneously added into the cell cultures Mg^{2+}-free medium they induced a significant increase of glutamate extracellular levels. Furthermore, NT (0.1 nM) significantly amplified the NMDA (0.1 μM)-induced increase in glutamate levels (Fig. 2A). The amplification of the NMDA receptor signalling induced by the presence of a threshold concentration of NT, was mediated by the activation of the NTS1 receptor subtype, since the pre-treatment with the selective NTS1 receptor antagonist SR48692 counteracted the enhancement induced by the co-application of NT and NMDA at the concentrations used (Fig. 2B; Antonelli et al., 2004). To strengthen the hypothesis for a possible existence of an interaction between NT and NMDA receptors, it remains to be established whether also the pre-treatment with the NMDA receptor antagonist MK-801 (1 μM) counteracts the enhancement of glutamate levels induced by the co-application of NT and NMDA. However, it is worth noting that at higher concentration (10 nM) NT induced by itself an increase in cortical glutamate levels and this effect was partly counteracted when MK-801 was added to the medium 10 min before the peptide (Fig. 3). As expected, the selective NTS1 receptor antagonist SR48692 fully counteracted the NT-induced increase in cortical glutamate levels (Fig. 3). The demonstration that the NT-

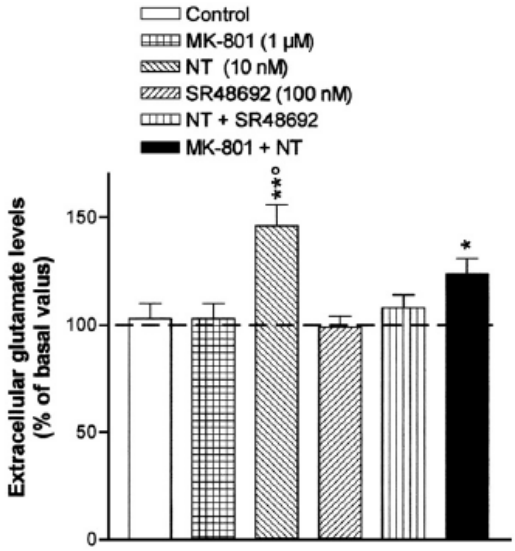

Fig. 3 – Effects of NT (10 nM), alone and in combination with SR48692 (100 nM) or MK-801 (1 μM), on extracellular glutamate levels in primary cell cultures of rat cerebral cortex. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.107 ± 0.006 μM), calculated from the mean of the first two fractions. NT was applied at the onset of the third fraction and maintained for 30 min, while MK-801 or SR48692 was added to the Mg^{2+}-free Krebs–Ringer solution 10 min or 20 min before the application of the peptide, respectively. Each column represents the mean ± SEM of 10 or 12 experiments for each group. *$p<0.05$; **$p<0.01$ significantly different from control, MK 801, SR48692 and NT + SR48692; °$p<0.05$ significantly different from MK 801 + NT according to ANOVA followed by the Newman–Keuls test for multiple comparisons.

induced increase on cortical glutamate levels is in part antagonised by the blockade of NMDA receptors further strengthens the hypothesis that the enhancement of glutamate outflow induced by the peptide is in part mediated by an involvement of an interaction between NT and NMDA receptors.

From a mechanistic point of view, it is worth noting that the PKC 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 (Antonelli et al., 2004). According to this possibility, Skeberdis et al. (2001) demonstrated the existence of a mGluR1-mediated potentiation of NMDA receptors that involved the activation of PKC. Thus, considering all these *in vitro* findings it may be postulated that the NT-induced increase of glutamate transmission, associated with a PKC-induced amplification of NMDA receptor signalling, could represent one of the main mechanisms underlying the potential neurodegenerative action of NT.

3.1.2. In vivo NT/NMDA receptor interactions studied on cortical and striatal extracellular glutamate levels of awake rats

Besides the well documented modulation of the DA transmission by NT (Nemeroff and Cain, 1985; Deutch and Zahm, 1992; Fuxe et al., 1992; Rostene et al., 1992; Sotty et al., 2000; Legault et al., 2002), more recent *in vivo* microdialysis experiments provide evidence that NT and its biologically active carboxy-terminal fragment NT(8–13) directly enhance the neuronal activity of glutamatergic neurons in discrete rat brain regions of the basal ganglia such as the striatum, the substantia nigra and the medial prefrontal cortex (Sanz et al., 1993; Ferraro et al., 1995, 1998, 2001). The observation that these effects are counteracted by the co-administration of the specific NTS1 antagonist SR48692 suggests that the NT-induced effects on glutamatergic transmission are mainly mediated by the activation of local NTS1 receptors.

In line with the data obtained in cortical cell cultures (Antonelli et al., 2004; see above) *in vivo* microdialysis studies demonstrated that local perfusion with a concentration of NT (by itself ineffective) (10 nM) enhances the glutamate releasing action of NMDA (100 μM) both within the cortex (*data not shown*) and the neostriatum (Fig. 4) of the awake rat. The NT-induced amplification of the NMDA receptor signalling is mediated *via* the activation of the NTS1 receptor subtype, since the NTS1 receptor antagonist SR48692 blocks the ability of the peptide to enhance the action of NMDA (Fig. 4B). In this context, it is worth noting that the presence in the perfusate

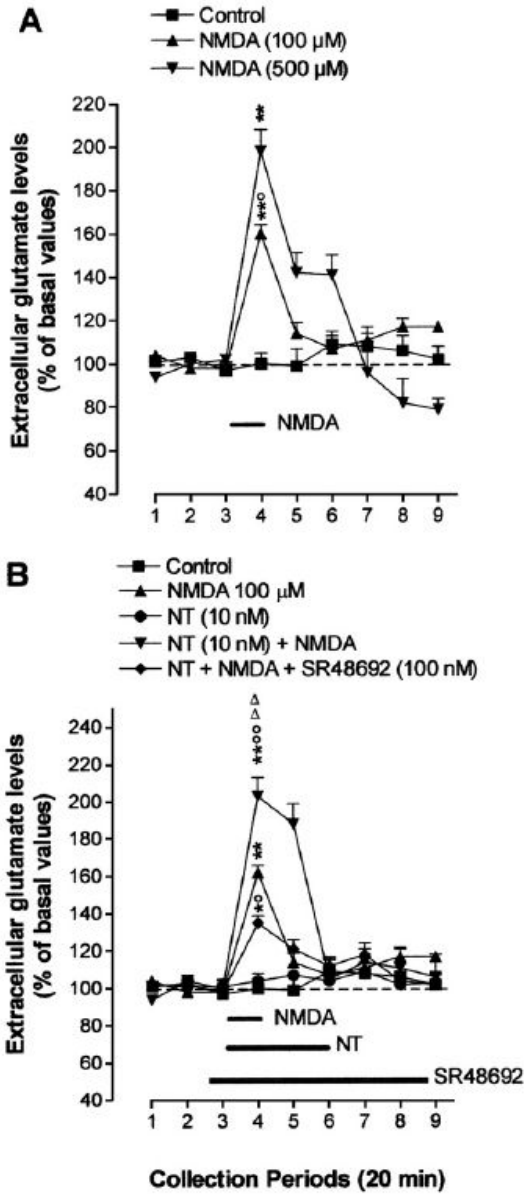

Fig. 4 – Effects of local perfusion with NMDA (panel A) and NMDA in the presence of NT (panel B) and NT plus SR48682 on extracellular glutamate levels from the striatum of the awake rats. The animals were implanted with a microdialysis probe into dorsolateral striatum (coordinates relative to the bregma: A:+0.8; L:±3.5; V:−5.5). Following the implantation, the probe was permanently secured to the skull and 36 h later the release experiment was performed. On the day of the experiment, the probe was perfused with Ringer solution. The collection of perfusate samples started 300 min after the onset of perfusion to achieve stable dialysis glutamate levels and perfusates were collected every 20 min. After three stable basal values were obtained, NMDA was locally perfused for 15 min while NT was perfused into the striatum for 60 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 6–8 animals. Panel A: $^{**}p<0.01$ significantly different from control; $^{\circ}p<0.05$ significantly different from NMDA 500 μM. Panel B: $^{*}p<0.05$; $^{**}p<0.01$ significantly different from control as well as NT (10 nM); $^{\circ}p<0.05$; $^{\circ\circ}p<0.01$ significantly different from NMDA 100 μM; $^{\Delta\Delta}p<0.01$ significantly different from NT + NMDA + SR48692 according to ANOVA followed by the Newman–Keuls test for multiple comparisons.

medium of SR48692 (100 nM) reduces the NMDA (100 and 500 μM)-induced increase of extracellular glutamate levels (Fig. 5). Such an effect may be due to the fact that NMDA perfusion causes a rise in endogenous NT levels (Radke et al., 2001), which might contribute to the NMDA-induced ability to enhance glutamate transmission. Thus, from these *in vivo* findings it seems likely that SR48692 counteracts this "neurotensinergic" dependent part of the NMDA effect.

In summary, these *in vitro* and *in vivo* findings show that NT increases glutamate release and simultaneously amplifies the glutamatergic receptor signalling, in particular the responsiveness of the NMDA receptors. These double activations are especially important for the toxic actions of glutamate and strengthen the hypothesis for a pathophysiological role of NT in glutamate-induced neurodegeneration.

4. Morphological and neurochemical evidence for the involvement of NTS1 receptors in the enhancement of glutamate-induced excitotoxicity in mesencephalic and cortical neurons in primary cultures

According to the above postulated hypothesis an involvement of NT in modulating glutamate excitotoxicity has been demonstrated in primary cultures of mesencephalic dopaminergic (Antonelli et al., 2002) and cortical (Antonelli et al., 2004) neurons. In particular, evidence has been obtained indicating that NT-induced increase of glutamate transmission in these brain regions may represent one of the triggering events responsible for the observed degenerative effects of the peptide on dopaminergic mesencephalic and cortical neurons (Antonelli et al., 2002, 2004).

The vulnerability of cultured mesencephalic dopaminergic neurons and cortical neurons to excitotoxic injury of glutamate was assessed by exposing the cells to the aminoacid. The cell damage induced by the toxic exposure was evaluated by analysing biochemical ([^{3}H]DA and [^{3}H]GABA uptake) and morphological (tyrosine hydroxylase-immunoreactive cells and Hoechst 33258 nuclear staining) parameters. Since the activity of the DA and GABA uptake systems in mesencephalic and cortical cell cultures can be considered as an index of metabolic as well as structural integrity of the dopaminergic and cortical neurons in culture, the determination of the [^{3}H]DA and [^{3}H]GABA uptake was used as a biochemical parameter to evaluate the glutamate-induced cell damage (Antonelli et al., 2002; Tomasini and Antonelli, 1998). Thus, it has been demonstrated that the neurotoxic effect of glutamate on the dopaminergic and cortical neurons was exacerbated by the presence of NT (Antonelli et al., 2002, 2004). In fact when the peptide (in threshold concentration) was applied in combination with glutamate, NT induced a further and significant concentration-dependent decrease of [^{3}H]DA and [^{3}H]-GABA uptake with respect to that observed with the aminoacid alone. The enhancing action of NT on glutamate-induced toxicity in dopaminergic neurons was also demonstrated by the increased disappearance of TH-immunoreactive neurons in those mesencephalic cultures pre-treated simultaneously with glutamate (100 μM) and NT at a concentration ineffective by itself (10 nM) (Antonelli et al., 2002). Such an effect was

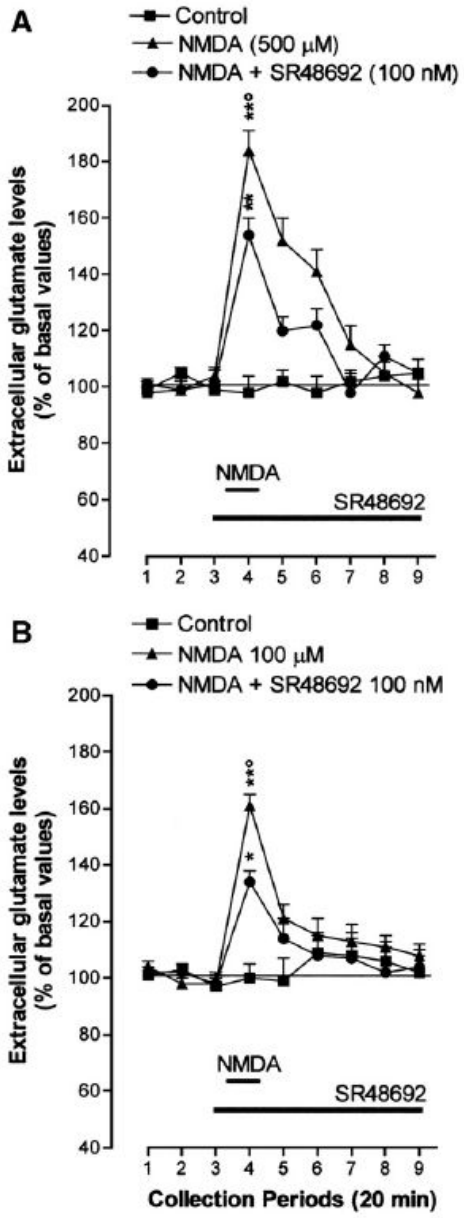

Fig. 5 – Effects of local perfusion with NMDA 500 μM (panel A) and 100 μM (panel B) in the presence of the NTS1 receptor antagonist SR48692 on extracellular glutamate levels from the striatum of the awake rats. NMDA was locally perfused for 15 min while SR48692 was perfused into the striatum for 120 min, as indicated by the respective solid bars. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. For further experimental details see Fig. 4 legend. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 4–8 animals. Panel A: **$p<0.01$ significantly different from control; °$p<0.05$ significantly different from NMDA+SR48692 group. Panel B: $p<0.05$, **$p<0.01$ significantly different from control; °$p<0.05$ significantly different from NMDA+SR48692 group, according to ANOVA followed by the Newman–Keuls test for multiple comparisons.

confirmed in cultured cortical neurons since the treatment with 10 nM NT (by itself ineffective) in combination with glutamate increased the number of apoptotic nuclei (Antonelli et al., 2004). The selective non peptide NTS1 receptor antagonist SR48692 (100 nM) counteracted the action of NT on glutamate-induced effects on dopaminergic and cortical neurons, suggesting that NTS1 receptors play a key role in the NT-induced potentiation of glutamate neurotoxicity. The evidence that NT participates in glutamate-mediated excitotoxic processes, leading to general death and/or apoptosis in various cultured neurons, gives further evidence of a possible relevant pathophysiological role for NT in glutamate-induced neurodegeneration.

5. Relevance of the NT neuron systems in 6-OHDA-lesioned rats

Moving from the above observations and in order to further investigate the possible relevance of NT in neurodegenerative process *in vivo*, a new set of experiments has been recently performed. The postulated neuroprotective effects of pretreatment with an antagonist of NTS1 receptors (SR48692) have been tested in a rat model of Parkinson's disease [unilateral nigral 6-hydroxydopamine (6-OHDA) induced lesion of the nigrostriatal DA pathway, hemiparkinson model]. Behavioural and biochemical experiments have been carried out in different groups of animals: sham-operated rats and 6-OHDA unilaterally lesioned rats chronically treated with saline or with the NTS1 receptor antagonist.

5.1. *Effects of chronic treatment with SR48692 on apomorphine-induced contralateral turning behaviour in hemiparkinson rats*

Animals unilaterally lesioned by an injection of 6-OHDA (8 μg dissolved in 4 μl of a 0.02% ascorbic acid saline solution) into the right substantia nigra, were divided into three groups: untreated rats and rats treated with the NTS1 receptor antagonist SR48692 (0.1 mg/kg i.p.) or its solvent (0.01% DMSO–saline solution: vehicle) from one-week before until one-week after the lesion. The same protocol of treatments was followed in sham-operated rats. At 1, 2 and 3 weeks after the lesion, the turning behaviour of animals in response to apomorphine was tested. As expected, in 6-OHDA-lesioned rats the apomorphine injection (0.1 mg/kg s.c.) produced a contralateral turning behaviour that significantly and progressively increased from week 1 to the 3rd week following the lesion. However, interestingly, in the SR48692-treated group, but not in the vehicle-treated group, the apomorphine-induced rotational behaviour is significantly reduced at each time of evaluation (days 7, 14 and 21 post lesion). Moreover, whereas the treatment stopped 2 weeks before, the effect of the compound remains significant (Fig. 6).

All groups of sham-operated animals failed to rotate in response to apomorphine injection. The findings of the present behavioural study indicate that systemic administration of NTS1 antagonist decreased the functional consequence of a partial dopaminergic lesion induced by intranigral application of the neurotoxin 6-OHDA in the rat.

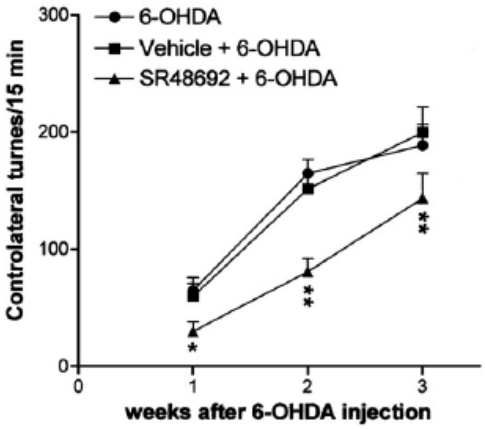

Fig. 6 – Turning behaviour (total turns contralateral to the lesioned side per 15 min) induced by apomorphine (0.1 mg/kg, s.c.) in 6-OHDA-lesioned animals chronically treated with SR48692 (0.1 mg/kg) or its vehicle. Results are expressed as mean ± SEM. (n = 14–15 per treatment). *p < 0.05; **p < 0.01 significantly different from 6-OHDA as well as vehicle + 6-OHDA groups according to repeated measures ANOVA followed by Newman–Keuls test for multiple comparisons.

5.2. *Effects of chronic treatment with SR48692 on unilateral nigral 6-OHDA-induced modifications of striatal glutamate levels*

In view of the above behavioural findings, a preliminary set of neurochemical experiments have been carried out in sham-operated animals and in rats chronically treated (i.p. administration) with SR48692 or its vehicle from one-week before until one-week after the 6-OHDA injection. In particular, the responsivity to a challenge with NMDA has been assessed.

In the groups of 6-OHDA-lesioned control and vehicle-treated rats, intrastriatal perfusion (10 min) with NMDA (100 μM) induced a slight increase (122 ± 3% of basal values) in glutamate extracellular levels that was significantly lower than that observed in sham-operated animals (160 ± 6% of basal values). Interestingly, in 6-OHDA-lesioned rats chronically treated with SR48692, the effect of intrastriatal perfusion of NMDA (100 μM) induced an increase (141 ± 5% of basal values) in glutamate extracellular levels that was significantly higher with respect to that obtained in the group of 6-OHDA-lesioned rats but still lower to that observed in sham-operated rats. Any changes in the responsiveness to NMDA challenge were observed in all groups of sham-operated animals.

6. Concluding remarks

In the above reported preliminary studies, behavioural and functional parameters have been evaluated in order to investigate the putative neuroprotective effects of SR48692 (systemically administered) in an *in vivo* animal model of Parkinson's disease (unilateral nigral 6-OHDA lesion). The results obtained suggest that the NTS1 receptor antagonist partially protects

dopaminergic neurons from 6-OHDA-induced degeneration. The above observation seems to be supported mainly by the behavioural data indicating that chronic treatment with the NT receptor antagonist significantly decreased the apomorphine-induced contralateral rotations in 6-OHDA-lesioned animals, a behaviour that have been well correlated with the degree of DA denervation (Schwarting and Huston, 1996).

The mechanism of action of SR48692 in these lesioned animals should be difficult to interpret taking into account both the role of NT in DA, glutamate and GABA release and the feed-back loops between the different brain regions involved in the extrapyramidal system. However, our first neurochemical observations are encouraging, demonstrating that a treatment with a specific NTS1 receptor antagonist, partially but significantly counteracts the degenerative process induced by 6-OHDA in the nigrostriatal pathway.

The neurochemical results are in line with previous microdialysis data (Marti et al., 2005) indicating that DA denervation is associated with a reduction of the enhancement of striatal glutamate transmission induced by a high micromolar NMDA concentration. Since it has been demonstrated that endogenous DA facilitates strong excitatory inputs in the striatum (Marti et al., 2005), the reduction of NMDA-stimulated glutamate levels in lesioned animals could be attributed to a loss of facilitatory DA receptor mediated signals (Marti et al., 2002). In view of above, the observation that in rats chronically treated with SR48692 the excitatory response to a NMDA stimulus is partially restored on the striatal glutamatergic transmission may support a protective action of the NTS1 antagonist against 6-OHDA-induced DA neuron degeneration.

The primary target for the neuroprotective action of SR48692 on DA nerve cells is likely to be its antagonistic action at NTS1 receptors leading to a partial protection or restoration of the dopaminergic nigrostriatal transmission as evidenced by the decrease in apomorphine-induced turning after intranigral injection of 6-OHDA. Indeed, as reported above, the activation of NTS1 by NT significantly enhances glutamatergic signalling in different brain areas including the substantia nigra and amplifies the NMDA receptor signalling (Antonelli et al., 2004). This suggests a reinforcing action of NT not only on several functions exerted by glutamate in the central nervous system, but in particular on the glutamate-mediated excitotoxicity, as demonstrated in primary cultures of mesencephalic DA (Antonelli et al., 2002) and cortical (Antonelli et al., 2004) neurons. In view of these findings and the evidence that 6-OHDA lesions of DA neurons induced an increase in NT levels in the basal ganglia (Zahm and Johnson, 1989; Taylor et al., 1992; Martorana et al., 2003), it seems likely that NT by enhancing excitotoxic signals could contribute to DA neuronal cell loss and that SR48692 exerts its neuroprotective action by counteracting NT effects at NTS1 receptors. Thus, the treatment with SR48692 in lesioned rats, by preventing the loss of dopaminergic inputs on the striatum, may lead to a further protection of the cortical glutamatergic inputs on the striatum, as evidenced by the partial increase in striatal glutamate level.

In conclusion, the present neurochemical, biochemical and behavioural data suggest that the use of selective NTS1 receptor antagonists in combination with conventional drug treatments likely provides a possible novel therapeutic approach especially for the treatment of Parkinson's disease, underlined also by the high densities of NTS1 receptors found in the nigrostriatal DA cells.

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