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Mechanisms of glia-mediated neurodegeneration: modulation by Nerve Growth Factor (NGF) and the purinergic system

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Riassunto

L'obiettivo del progetto di tesi è stato quello di comprendere i meccanismi di neurodegenerazione mediati dalle cellule gliali e caratterizzare gli eventi molecolari implicati nella neuroprotezione da parte del Nerve Growth Factor (NGF).

L'interesse per le cellule gliali si basa sul nuovo concetto di sinapsi tripartita che afferma l'esistenza di una comunicazione bidirezionale tra neuroni e astrociti. Per questo studio, è stato utilizzato un modello di gliosi reattiva in-vitro basato su popolazioni pure di astrociti e/o microglia attivati mediante trattamento con Lipolissacaride (LPS) o con la citochina pro- infiammatoria Tumor Necrosis Factor-α (TNF-α). Inizialmente, questo sistema è stato usato per studiare gli effetti dell'attivazione gliale sulla proliferazione e valutare la potenziale attività anti-gliosi del NGF. Esperimenti di curva di crescita e di incorporazione di BrdU mostrano che sia LPS che TNFα determinano un significativo aumento della proliferazione astrocitaria, che viene ridotto dal co-trattamento con NGF. Da un punto di vista molecolare, sono stati studiati i livelli di espressione dei recettori: TrkA e p75. In effetti, i dati ottenuti indicano che i trattamenti con LPS aumentano i livelli di espressione di p75 e diminuiscono i livelli di TrkA. Questo effetto è inibito dal cotrattamento con NGF.

Contemporaneamente è stato studiato l'effetto dell'attivazione gliale su sopravvivenza e funzionalità neuronale utilizzando un modello di neuroni corticali esposti a medium condizionato (CM) da astrociti attivati in

seguito a trattamento con LPS o TNFα. In effetti, il trattamento con il CM da astrociti attivati diminuisce la sopravvivenza dei neuroni e riduce significativamente i livelli di TrkA, ma non in presenza di NGF. Inoltre, il NGF inibisce l'aumento di ROS indotto dal trattamento con CM da cellule gliali attivate con TNFα.

Un potenziale meccanismo di tossicità potrebbe essere mediato dall'aumento di pro-NGF (precursore del NGF) e di metalloproteinasi-9 (MMP-9), responsabile della maturazione e della degradazione del NGF. L'analisi RT-PCR, inoltre, ha portato all'identificazione di diversi geni associati alla funzionalità sinaptica e che sono down- o up-regolati dal trattamento con CM o con NGF. Infine, è stato valutato l'effetto del NGF sulla funzionalità mitocondriale degli astrociti. Esperimenti preliminari, effettuati tramite Seahorse, indicano che il NGF aumenta la capacità respiratoria degli astrociti sia in condizioni basali che in condizioni di stress.

Contemporaneamente sono stati svolti alcuni studi sul ruolo del sistema purinergico nella neuroinfiammazione. L'effetto del sistema purinergico sull'attivazione degli astrociti è stato valutato tramite OxATP, antagonista dei recettori P2X7. I dati mostrano che il co-trattamento cronico con OxATP previene in maniera significativa la proliferazione degli astrociti. Inoltre, il trattamento con OxATP non ha effetto sulla sopravvivenza neuronale e astrocitaria, ma previene la produzione di ROS. Infine, OxATP inibisce la riduzione dei livelli di vGLUT, il trasportatore del glutammato, sia nei neuroni che negli astrociti attivati.

Nel complesso, questi studi identificano due meccanismi di neurodegenerazione mediata dalla glia: uno legato ad alterazione del sistema di supporto neurotrofico da parte del NGF, l'altro legato ad eccessiva attivazione del sistema purinergico.

Summary

The objective of my thesis project was to understand mechanisms of neurodegeneration mediated by glial cells and characterize the molecular events involved in neuroprotection by Nerve Growth Factor (NGF).

The interest in glial cells is based on the new concept of tripartite synapses and the existence of a bidirectional communication between neurons and astrocytes. For this study, we used a model of in-vitro reactive gliosis based on pure populations of astrocytes and/or microglia activated by Lipopolysaccharide (LPS) or the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNFα). Initially, this system was used to study the effects of glial activation on astrocytes proliferation and evaluate the anti-gliosis activity of Nerve Growth Factor (NGF). Experiments of growth rate and BrdU incorporation showed that both LPS and TNFα determined a significant increase in astrocytes proliferation, which was reduced by co-treatment with NGF. At molecular level, we studied expression levels of NGF receptors: TrkA and p75. Our data indicated that treatments with LPS increased expression levels of p75 and decreased TrkA levels. This effect was prevented by co-treatment with NGF.

We also studied the effects of glial activation on neuronal survival and functions by using a model of cortical neurons exposed to conditioned medium (CM) from activated astrocytes following treatment with LPS or TNFα. In fact, treatment with the CM from activated astrocytes decreased

the survival of neurons and significantly reduced the levels of TrkA, but not in the presence of NGF. It was also seen that CM from glial cells activated with TNFα, induced a significant increase of ROS, that was completely inhibited by pre-incubation (ON) with NGF.

A potential mechanism of toxicity may be mediated by pro-NGF (precursor of NGF), through the increase of metalloproteinase- 9 (MMP-9), the main protease responsible for maturation and degradation of NGF. RT-PCR analysis also led to the identification of a series of genes that participate to synaptic function and are down- or up-regulated by the treatment with CM and NGF. Finally, we assessed the effect of NGF on astrocytic mitochondrial function. Preliminary experiments, by Seahorse, indicated that NGF increased the respiratory capacity of astrocytes both in basal conditions and under stress conditions.

In parallel, we studied the role of the purinergic system in neuroinflammation. Indeed, several studies have shown that activation of P2X7 receptors (a class of purinergic receptors), induces the secretion of pro-inflammatory cytokines and chemokines, such as TNFα, and also stimulates the production of superoxides in microglial cells. The effect of the purinergic system on glial activation was assessed by using OxATP, a P2X7 receptor antagonist. Our data showed that chronic co-treatment with OxATP significantly prevented the proliferation of astrocytes. Furthermore, treatment with OxATP had no effect on neuronal survival and astrocytes, but prevented the production of ROS. Finally, OxATP

inhibited the decrease of astrocytic and neuronal vGLUT, the glutamate transporter, both in neurons and activated astrocytes.

In conclusion, these studies identify two main mechanisms of glia-mediated neurodegeneration: one based on the alteration of neurotrophic support by NGF, the other due to activation of the purinergic system.

INTRODUCTION

Cells of the Nervous System

The brain, the most complex organ in all Vertebrates, has to function over a long time at the same time adapting to environmental changes. As a result, the brain circuitries need to be exceptionally plastic, and indeed many brain areas (for example hippocampus or the visual cortex) are prone to a constant remodelling. Other brain regions, responsible for vital functions, such as breathing, should rigidly adhere to a conserved structure. This implies the need of autoregulatory systems, which control all aspects of brain development and function. To achieve this, the brain is isolated from the rest of the body by the blood–brain barrier (Giaume et al., 2007). The overall functionality of the brain is based on the dense network of interconnections established between different cell type, each playing a specif role on synaptic formation and function.

Neurons

Neurons represent the main cell unit of the Nervous system. They are cells specialized in the reception of stimuli and in the conduction of impulses from nerves. They are essential for the transmission of information to other parts of the body. Number and size of neural cells increase with the size of the body and of the brain. This increasing quantity has caused the generation of a new quality, the intellect (Giaume et al., 2007).

Although it is possible to classify neurons from a morphological point of view, as well as for functional and cytochemical properties, each neuron consists of:

- **Soma (cell body)**, in which are contained the nucleus and other cytoplasmic organelles responsible for cellular metabolism and the synthesis of neurotransmitters. The soma is also the region responsible for integration of the signals collected from dendrites.
- **Dendrites** receive information from other neurons: they emerge from various points of the cell body and branch repeatedly. They are covered with specific structures (called spine) specialized in synapses formation.
- **Axon**, which originates from a protrusion of the soma (growth cone), is long and uniform. Axons are responsible for the passage of information, and the speed of nerve impulses depends of the diameter of the axon. The terminal part of the axon is defined synaptic button and is the site where contact is made with other neurons or other target cells. The contact point is called synapse.

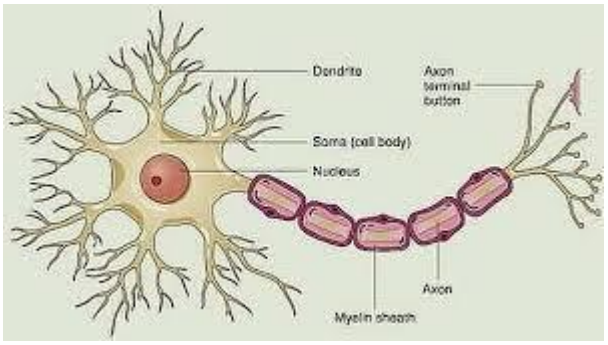


Figure 1.1. Structure of neuronal cell (Wiley and Sons, 2000)

Glial cells

Glial cells are the non-neuronal component of the brain. Different types of glial cells (astrocytes, microglia, oligodendrocytes or Schwan cells) play distinct functions in structural/trophic support, formation of myelin and protection of the brain.

The term “glia” (from the Greek word for “glue”) implied their main function in holding neurons together. However, the recent discovery of glial neurotrasmitters, receptors and transportes has led to the current knowledge about the role of glial cells in brain homeostasis and function.

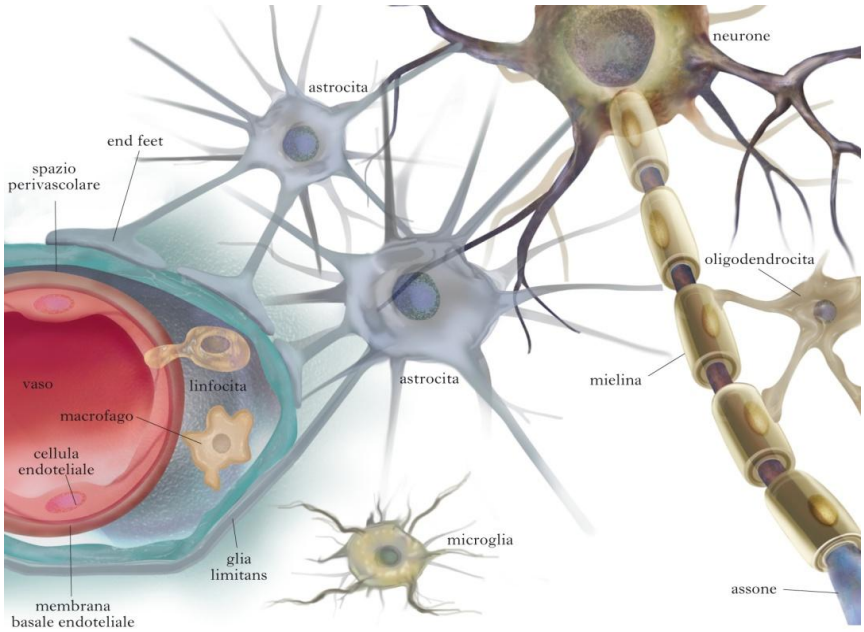


Figure 1.2. Cells of CNS

Schwann cells and Oligodendrocytes

Schwann cells and oligodendrocytes protect neuritis, as well as they increase the speed of nerve conduction. Schwann cells and oligodendrocytes are, in fact, responsible for the formation of the myelin sheath (in the peripheral nervous system and in the central nervous system, respectively) due to multiple concentric layers of their plasmamembrane around the axon and the gradual expulsion of cytoplasm. Each Schwann cell is able to enwrap a single internode of only one axon, while oligodendrocytes take contacts with more neurons. The myelin sheath, presents numerous interruptions defined “nodes of Ranvier”, where there is an effective passage of ions across the plasmamembrane. Then the impulse “jumps” from one node of Ranvier to another.

Microglia

Microglia cells the macrophages of the brain and costituite 10% of cells in the Central Nervous System. Microglial cells derive from primitive myeloid progenitors (which originate from the yolk sac) invading the Central Nervous System (CNS) during the embryonic development. As a consequence, they are the only immune cells that permanently reside in the brain. These surveillant microglia have a very branched morphology and can rapidly respond to any pathological stimulus by transforming their morphology and functional behavior. Traditionally, these changes in the microglial phenotype are referred as *microglial activation*.

Activated microglia loose their branchied morphology and acquire the capability to proliferate, migrate, and release reactive oxygen species (ROS), neurotoxins, and pro-inflammatory and anti-inflammatory cytokines. These activated microglia can secrete trophic factors, present antigens to T cells, and are capable of phagocytosis to remove pathogens, degenerating cells, and inflammatory debris. Surveillant microglia can also directly contact synaptic elements and eliminate particular subsets of axonal terminals and dendritic spines, depending on changes in neuronal activity and sensory experience, both in the developing and mature brain. Gene expression and morphological changes associated with microglial activation have been extensively studied. Indeed, activated microglia was found to eliminate neuronal precursors, and to regulate the density of dendritic spines and the functional maturation of glutamatergic receptors.
In addition, in the mature CNS, surveillant microglia was also found to remove newborn cells during adult hippocampal neurogenesis and regulate glutamatergic synaptic transmission in the hippocampus (Siskova et al., 2013). As the microglia is able to influence neuronal activity, in the same way, also neurons can inhibit, for example, microglial activation through both receptor ligand interactions and secreted molecules, such as fractalkine (CX3CL1), which act on the microglial receptor CX3CR1. Derepression of these inhibitory signals is known to promote microglial activation. Recently, two proapoptotic caspases, capase-8 and caspase-3/7, were shown to promote microglial activation downstream of Toll-like receptor 4 (TLR4) in vitro. Moreover, pharmacological inhibition of

caspase-3/7 suppressed the induction of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and tumor necrosis factor–a (TNFα), and provided modest protection against dopaminergic neuron loss (Aguzzi et al., 2013).

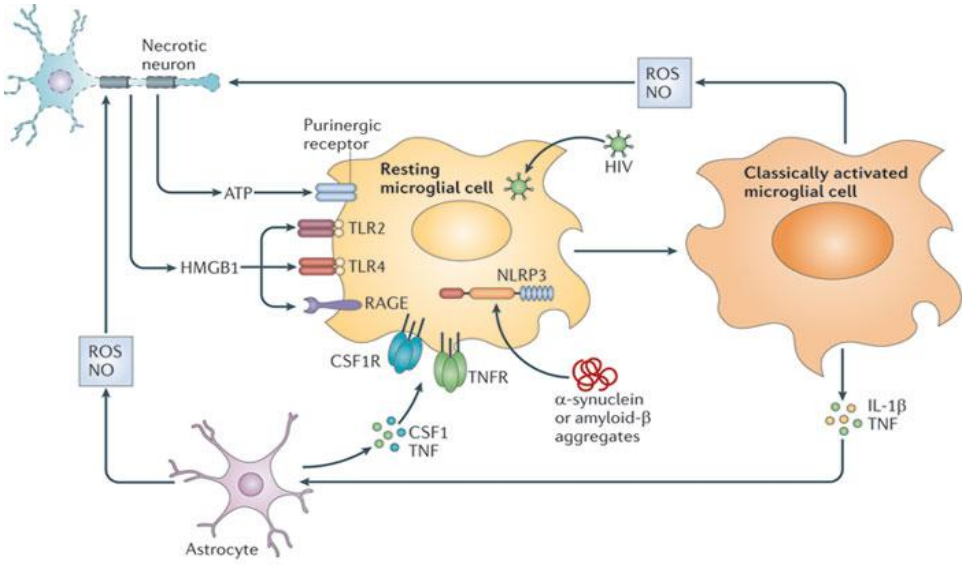


Figure 1.3. Model of glial activation

Astrocytes and Tripartite Synapse

Astrocytes are the predominant cells in the Central Nervous System. This name originates from their characteristic shape of a star with a central cell body and long processes that extend in all directions. Astrocytes are multifunctional cells that might well be considered the “cornerstone” of

brain cytoarchitecture and function. In addition to their trophic and structural role, astrocytes are dynamic components of brain connectivity and function (Colangelo et al., 2014). The complexity of cellular circuitries in the CNS resides in the dynamic changes of neural connections (i.e. synaptic plasticity). This function, through a highly developed blood– brain barrier, is entrusted to glial cells. In particular, astrocytes provide for the micro architecture of the gray matter by forming relatively independent structural domains. Within these domains, each astrocyte covers synaptic contacts (Tripartite Synapse) and establishes connections with neuronal membranes and blood vessels. Through gap junctions, astrocytes of distinct domains create an astroglial syncitium, thus providing a glial information-transfer system, a pathway for rapid intercellular diffusion and long-range signaling.

Several studies support the key role for astrocytes from synaptic formation to metabolic support and neurotransmitter release.

Astrocytes represent, also, the key elements in synaptogenesis. It has been reported that addition of astrocytes to in vitro neuronal cultures triggers a significant increase in synapse formation: through production of cholesterol and release of trophic factors, astrocytes are crucial for synapse maturation and maintenance; through production and release of thrombospondins 1 and 2, they promote synaptogenesis suggesting their crucial role in post-lesion synaptic plasticity, remodeling and regeneration. Astrocytes are also essential for neuronal energy metabolism and glutathione synthesis. Astrocytic endfeet contact

neighboring capillaries through perivascular processes, thus forming a functional link between neurons and blood vessels. An increase in neural activity within an astrocytic domain results in the release of vasoactive substances (arachidonic acid metabolites) that promote dilation in nearby arterioles. Metabolic support to neurons is achieved through the astrocyte–neuron lactate shuttle. Astrocytes convert glucose to lactic acid, which is then taken up into neurons and converted to pyruvate for energy metabolism. Thus, astrocytes play a central role in coupling synaptic plasticity and glucose metabolism (neurometabolic coupling) through mechanisms involving the sodium-coupled glutamate re-uptake, which stimulates aerobic glycolysis and production/release of lactate. Neurometabolic coupling is a central physiological principle of brain function that has provided the basis for 2-deoxyglucose-based functional imaging with positron emission tomography (PET) (Magistretti et al., 2006). The metabolic function of astrocytes is supported by a large number of studies. More recently, astrocytic glycogen breakdown and astrocyte-neuron lactate transport have been demonstrated to be essential for long-term memory formation, and for the maintenance of long-term potentiation (LTP) of synaptic strength.

The different metabolic requirements of astrocytes and neurons (high and low glycolytic, respectively) is based upon their different enzymatic assets. This mechanism appears to be linked to the neuronal need to redirect glucose metabolism to the pentose-phosphate (PPP) pathway to generate NADPH, a necessary cofactor in the regeneration of reduced

glutathione (GSH). Neurons are particularly vulnerable to reactive oxygen (ROS) and nitrogen species (RNS), and also have low activity of γ-glutamyl cysteine synthetase, the limiting enzyme for GSH synthesis. Therefore, neuronal downregulation of glycolysis and glucose metabolism through the PPP pathway seem to be required to maintain their antioxidant status.

Another important function of astrocytes is to control the concentration of ions. In fact, extracellular K^+ accumulated from neural activity is lowered by inward rectifying K^+ channels (Kir-channels) controlling ion concentration.

In order not to alter the neuronal stimuli, it is very important to maintain a balanced concentration of neurotransmitters in the brain. In this regard, another key function of astrocytes is, moreover, the removal of neurotransmitters that are released by active neurons, in particular glutamate. Glutamate, the main excitatory neurotransmitter in CNS, is also the most powerful neurotoxin when it accumulates in the extracellular space. From the bulk of glutamate released during synaptic transmission, about 20% is re-captured by postsynaptic neurons, the remaining 80% is taken up by perisynaptic astrocytic. Astrocytes selectively express two glutamate transporters, EAAT1 and EAAT2 (in rodents known as GLAST and GLT1, respectively).

Besides their role in glutamate uptake, astrocytes are also crucial for the recovery of glutamate in presynaptic terminals through the glutamate–glutamine shuttle system. Astrocytic cytosolic glutamate is converted by

the astrocytic-specific glutamine synthetase into the non toxic glutamine, that is released in the extracellular space and after entering the neuronal compartment is converted into glutamate to reconstitute the neurotransmitter pool.

The ability of astrocytes to release chemical transmitters (gliotransmitters) is also fundamental for their involvement in neuro-glial networks. Among gliotransmitters, astrocytes can release glutamate, ATP, D-serine, GABA and other molecules through a Ca^{2+}dependent exocytosis or diffusion through either large pore channels (P2X7 receptors), transporters (glutamate transporters), or the cystine-glutamate antiporter system. This function strengthens the double role of astrocytes in the Tripartite Synapse: astrocytes sense neuronal activity and neurotransmitter release through the expression of neurotransmitters receptor on astrocytic membrane; on the other hand, they modulate the efficacy of the synapse by releasing gliotransmitters that in turn might modulate the strength of inhibitory or excitatory synaptic transmission through activation of neuronal receptors (Colangelo et al., 2012).

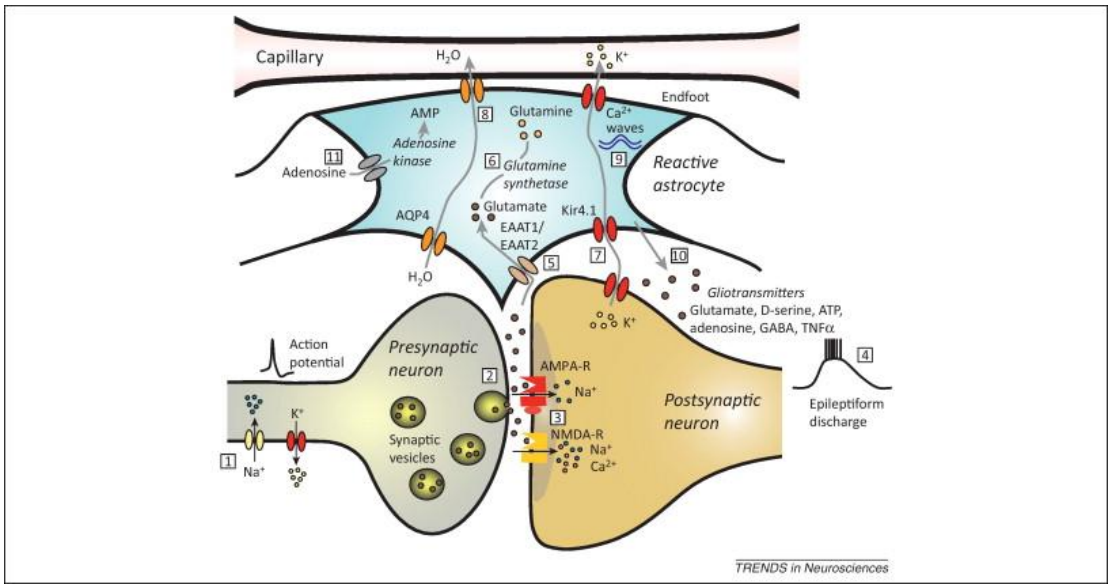


Figure 1.4. Scheme of the tripartite synapse (Devinsky et al., 2012)

Reactive gliosis

The activation of microglia and astrocytes, and the accompanying elaboration of pro-inflammatory mediators, occurs in the CNS of patients with diverse diseases. The level of glial cell reaction to a great extent reflects the severity of a brain injury. Innate immunity by antigen-presenting cells is the first line of defense against foreign materials. In the brain, this response is mainly orchestrated by microglial cells. In the presence of pathogens, acute neuronal insults, and more chronic neurological diseases, neuronal loss activates microglial cells in the CNS. As the primary immune effector cells of the CNS, activated microglial cells phagocyte the proteins of dead neurons, present this neuronal

fingerprint at their surface, and produce pro-inflammatory cytokines and toxic molecules that compromise neuron survival. As the first line of defense in the CNS, microglia must respond immediately to the presence of danger signals, react quickly to increased inflammatory signals and destroy the infectious agents before they damage sensitive neural tissue. Since this process must be done quickly to prevent potentially fatal damage, microglia are extremely sensitive to even small pathological changes in the CNS.

In contrast to the rapidly occurring microglial response, the astrocytes response is usually delayed. It is suggested that in conjunction with the secretion of cytokines, activated microglia disturb astrocyte functions and may, as a consequence, contribute to the subsequent activation of astrocytes. Microglial activation and astrogliosis were extensively examined by several studies both in-vivo and in-vitro. All the results of these studies suggested that microglial and astroglial reactions, which occur in response to neuronal death, are separated in time, with microglial activation preceding astrogliosis. This widely documented temporal relationship suggests that microglial activation might be involved in the onset and maintenance of astrogliosis (Zhang et al. 2013).

In addition, recent studies focused on the cellular and molecular mechanisms underlying astrogliosis suggest that pro-inflammatory cytokines and chemokines might play an important role as triggers and modulators of astrogliosis. Activated microglia are a predominant source of cytokines within the CNS and are reported to release a series of pro-

inflammatory cytokines and chemokines, such as interleukins, monocyte chemoattactant protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF), macrophage inflammatory protein-1α/β (MIP), TNF-α, etc. The levels of microglia-derived cytokines are known to be elevated following injury and the receptors for most of these cytokines have been identified on astrocytes. Astrocytes express receptors for IL-1 , IL-6 , IL-8, MIP, and M-CSF. Additionally, TNF-α receptors are reported to be constitutively expressed on astrocytes and have effects on glutamate transmission directly and indirectly by inhibiting glial glutamate transporters on astrocytes. Furthermore TNFα, appears to induce astrocyte proliferation and lead to increased expression of glial fibrillary acid protein (GFAP), important astrocytic marker.

In fact, astrogliosis is characterized by the increase of intermediate filaments with accompanying cellular hypertrophy and an abnormal apparent increase in the number of astrocytes. Reactive astrogliosis is highly conserved. The functions of reactive astrocytes are not well understood, and both harmful and beneficial activities are present in the literature. Upon activation, astrocytes upmodulate a large number of molecules and benefit the injured nervous system by regulating several biological processes. For istance, astrocytes play fundamental roles in maintenance and repair of the BBB (that consists of a series of structures collectively known as neurovascular units, which are composed of endothelial cells and astrocyte end feet separated by a basal lamina at their interface).

Astrocytes play fundamental roles in the formation of BBB, as well as in mechanisms involved in maintenance and repair of the BBB after CNS injury, such as those involving the secretion of factors that differentiate capillaries to the BBB type. In addition, astrocytes provide support and guidance for axonal growth and aid in improving functional recovery after CNS injury. However, prolonged activation of astrocytes becomes detrimental to axon growth. Hypertrophic astrocytic processes enmesh the lesion site and deposit an inhibitory extracellular matrix consisting primarily of chondroitin sulfate proteoglycans. This tissue reaction results in the formation of a dense complicated structure, named glial scar, that is inhibitory to regenerating axons. The glial scar formed mainly by reactive astroglia represents an inhibitory physical and chemical barrier for axonal regeneration and functional connection reestablishment.

Reactive astrocytes can produce pro-inflammatory and cytotoxic cytokines that are harmful to neurons or oligodendrocytes in the lesioned brain, which in turn can lead to further damage via, e.g., nitric oxide radicals and TNF-α. Expression of nitric oxide synthase is elevated in astrocytes in many neurological diseases. Nitric oxide produced by reactive astrocytes is able to damage local neural cells, oligodendrocytes and other cell types.

Astrogliosis is reported to be linked to the onset and duration of neural cell damage in the affected brain region and is believed to be an underlying component of a diverse range of diseases, including epilepsy, multiple sclerosis, amyotrophic lateral sclerosis etc. Neuronal damage

after acute traumatic brain injury also is closely linked to the formation of astrogliosis (Zhang et al., 2013).

Glial reaction involves activation of receptors, such as Toll-like receptors (TLR), transcription factors (NF-kB, Nrf2, AP-1, etc.) and signaling molecules (p38MAPK JNK, JAK/STAT3, etc.) of common inflammatory pathways, as well as alteration of protein expression (GFAP, vimentin, aminoacid transporters, receptors, etc.) and enzymes, like cycloxygenases (COX2). Nuclear translocation of NF-kB in reactive astrocytes was shown to mediate glial proliferation and inflammatory responses. Astrogliosis is responsible for the accumulation of excitotoxic levels of glutamate due to loss or dysfunction of astrocytic glutamate transporters. Excitotoxic levels of glutamate during neuroinflammation are also contributed by astrocytes through Ca^{2+}dependent secretory pathways following intracellular [Ca^{2+}] rise. Several astrocytes receptors are coupled to Ca^{2+}dependent signalings that can lead to glutamate release: prostaglandins (like PGE2), purinergic receptors, bradykinin and other GPCRs acting through Ca^{2+} mobilization from IP3R-sensitive intracellular stores. Purinergic receptor stimulation by ATP (released from damaged cells) can modulate several mechanisms of neuroinflammatory pathways, such as synthesis of cytokines, upregulation of COX-2, production of PGE2, proliferation, glutamate and GABA release, impairment of glutamate uptake, decreased expression/activity of glutamine synthetase (GS), increase of iNOS activity and NO production (Colangelo et al., 2014).

Phenotypic changes accompanied by modifications of Cx expression in astrocytes, as complex changes in Cx43 expression and gap junctional communication have been observed after brain injuries and pathologies known to be associated with reactive gliosis. During brain inflammation, microglial cells and astrocytes synthesise a variety of inflammatory mediators that regulate Cx43 expression and gap junctional communication in astroglia (Giume, 2007). In summary, reactive astrogliosis is one of the key components of the cellular response to CNS injury and it has been suggested to be an attempt by the CNS to restore homeostasis through isolation of the damaged region, while at the same time astrogliosis is commonly regarded as a major impediment to axonal regeneration. Reactive astrogliosis and scar formation might delay or inhibit regenerative responses, therefore, this astrocytic reaction may play an important role in the pathogenesis and progression of diverse neuropathological conditions.

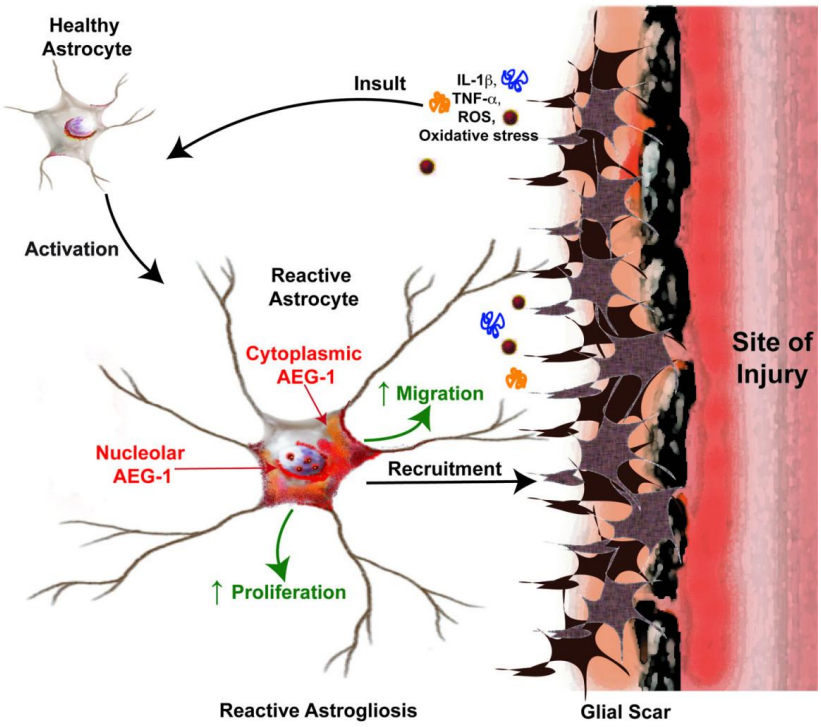


Figure 1.5. Schematic representation of reactive gliosis

Mitochondrial dysfunction

Mitochondria are essential organelles for a wide variety of cellular processes, including cell intermediate metabolism, calcium homeostasis, bioenergetics and intrinsic cell death processes. Mitochondrial dysfunction has long been associated with neurodegenerative diseases, because of the dependence on mitochondrial function of neuronal cells (Palomo et al., 2014). Mitochondria lie at the heart of energy provision for most cells and tissues, providing energy for the balance against

negative entropy, for maintenance of cell structure and for active signalling.

Structurally, mitochondria are compartmentalised by two membrane systems: the external outer mitochondrial membrane (OMM), the inter membrane space (IMS) and the matrix, which is enclosed by the inner mitochondrial membrane (IMM). The IMM is intensely but variably folded into cristae, which vastly expand the membrane surface area available for the bioenergetic transformations that are fundamental to mitochondrial function. The IMM houses the protein complexes of the electron transport chain, and provides a highly efficient and selective barrier to the flow of ions. The mitochondrial matrix contains the enzymes that drive the tricarboxylic acid cycle and beta-oxidation (Corona et al., 2014).

Mitochondria are the "powerhouses of the cell," the production of adenosine triphosphate (ATP), via the combined efforts of the tricarboxylic acid cycle and the respiratory chain/oxidative phosphorylation system (OxPhos). The respiratory chain is a set of biochemically linked multi-subunit complexes (complexes I, II, III, and IV), and two electron carriers (ubiquinone/coenzyme Q and cytochrome c). It uses the energy stored in food to generate a proton gradient across the mitochondrial inner membrane, while at the same time transferring electrons to oxygen, producing water. The energy of the proton gradient drives ATP synthesis via ATP synthase (complex V). ATP is then distributed throughout the cell.

The central importance of mitochondria for cellular energy production is underscored by the discovery of numerous syndromes resulting from OxPhos defects. The mitochondrial respiratory chain is the product of a joint effort between the mitochondrial and nuclear genomes.

Patients with OxPhos dysfunction who carry mutations in either mtDNA or nDNA present a number of clinical features, many of which are neurological, such as seizures, ataxia and cognitive impairment. The severity of the disorder correlate well with the degree of ATP deficit caused by the mutation. Thus, "mild" mutations could theoretically give rise to a slowly progressive, late-onset neurodegenerative disease, such as AD or PD. There are several nDNA-encoded and mDNA-encoded OxPhos-reated gene mutations associated with adult-onset neurodegeneration (Parkinsonism). For istance, in PD a meta-analysis of genome-wide gene expression microarray studies revealed the strongest association between PD and genes encoding for OxPhos subunits and for enzymes involved in glucose metabolism, all of which are regulated by PGC-1α, a transcriptional coactivator of mitochondrial biogenesis. Relevant to this observation is the identification of PARIS (Parkin-interacting substrate), a partner of the PD-related protein Parkin, that represses PGC-1α expression. Inactivation of Parkin, either by mutation or by environmental stress, leads to the accumulation of PARIS and the ensuing inhibition of PGC-1α transcription, which in turn may reduce mitochondrial biogenesis and cause OxPhos deficiency.

In neurons, mitochondria are enriched at presynaptic terminals at the ends of axons and at postsynaptic terminals at the ends of dendrites, where the bioenergetic demand is particularly high. In addition, while this constant motion helps the cell redirect and recycle mitochondria in an efficient manner, "worn-out" mitochondria are ultimately disposed of (and their component parts recycled) via autophagy ("mitophagy") or via extrusion of "mitochondria-derived vesicles" (Schon et al., 2011).

Given the critical role of mitochondria in maintaining cell viability, it stands to reason that defects in mitochondrial trafficking could underlie neurodegenerative processes. Such evidence, in fact, has been reported in autoptic samples from patients with sporadic AD: defects in axonal trafficking of molecular motor proteins and organelles, including mitochondria, were inferred from the observation of axonal swellings containing vesicles, vacuoles, multilamellar bodies, and especially mitochondria, in the nucleus basalis of Meynert; the formation of these vesicles was apparently mediated by the expression of kinesin-1, a microtubule motor.

On the other hand, ample data for trafficking defects exist in experimental models mainly genetically-engineered mice of a number of adult-onset neurodegenerative disorders. Both anterograd and retrograde mitochondrial transport were reduced in motor neurons from ALS mice expressing mutant superoxide dismutase-1 (SOD1) (Palomo et al., 2014).

More worth is the potential link between proteins known to cause familial PD and defects in microtubule-mediated trafficking. The mitochondrial

kinase PINK1 (PTEN-induced putative kinase-1), in fact, may play a role in mitochondrial transport. In addition, several studies in vitro show that upon overexpression of wild-type α-synuclein in differentiated SH-SY5Y neuroblastoma cells (which mimics the accumulation of the normal gene found in some PD patients), aggregates of the protein disrupted the microtubule network and microtubule-dependent trafficking of cargoes. Mitochondria do not exist, or operate, in isolation, but associate with many other subcellular organelles, such as peroxisomes, lysosomes, Golgi, and ER. Among these, the most intriguing is the connection between mitochondria and ER, that are linked, both biochemically and physically, via mitochondria-associated ER membranes (ER-MAM, or MAM). MAM has been reported to be enriched in several proteins, including those involved in calcium homeostasis. The relationship between MAM and calcium trafficking is worthy of some elaboration. The tethering of mitochondria to ER via MAM is a dynamic process, as organelles must disengage from the ER in order to engage, and then travel on, microtubules. Any defect that alters this equilibrium could conceivably result in a mismatch between the number of mitochondria required in specific regions of a neuron and the demand for mitochondrial cargo in those regions. Given the dynamic nature of MAM, and the role of IP3Rs in maintaining the proper equilibrium between ER and mitochondrial [Ca^{2+}], probably neurodegenerative disorders in which calcium homeostasis is disrupted could arise from altered ER-mitochondrial communication, or conversely, that alterations in calcium

homeostasis from some other cause could affect this communication indirectly. However, the most compelling case for a role for MAM in pathogenesis is familial AD due to mutations in presenilin-1 and -2, which are components of the γ-secretase complex that cleaves the amyloid precursor protein (APP) to produce amyloid-β, a constituent of the extracellular neuritic "plaques" that accumulate in the brains of AD patients. In a similar manner, "unwanted" mitochondria can be disposed of, and their contents recycled, by mitophagy.

Mutations in mitochondrial quality control genes could prevent the efficient elimination of damaged mitochondria and the degradation of superfluous and potentially deleterious polypeptides, therefore leading to neuronal dysfunction and perhaps ultimately to cell death. Mitochondria are deemed "bad" if they have a low $\Delta\psi$ and elevated ROS, indicative of defective OxPhos. It is becoming apparent that some genetic forms of Parkinson disease may be in essence disorders of mitochondrial quality control. Indeed, it seems that a loss of $\Delta\psi$ is a prerequisite for the disposal of bad mitochondria, the loss-of function mutations in DJ-1 that cause PD may impair mitochondrial quality control by altering the relationships among mitochondrial damage, $\Delta\psi$, and mitophagy (Schon et al., 2011).

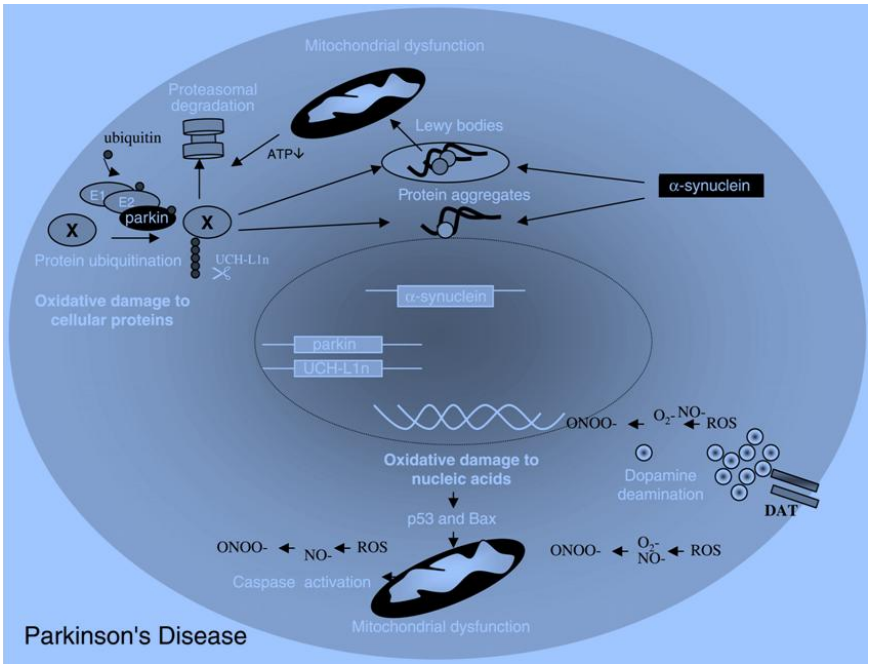


Figure 1.6. Example of mitochondrial dysfunction in PD (Mattison et al., 2012).

Neurotrophins

Neurotrophins were identified as proteins promoting of neuronal survival, but they also regulate many aspects of neuronal development and function, including synapse formation and synaptic plasticity. The first neurotrophin, Nerve Growth Factor (NGF), was discovered by Rita Levi Molntalcini during a search for factors that could explain the relevance of target tissues on the survival of motor and sensory neurons. Four neurotrophins are expressed in mammals: NGF, Brain-derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4). This class of proteins has been the focus of numerous studies,

which show that neurotrophins are able to interact and activate different receptors. To these receptors appears to be linked the ability of neurotrophins to regulate almost all aspects of neuronal development and function, including precursor proliferation and commitment, cell survival, axon and dendrite growth, membrane trafficking, synapse formation and function, as well as glial differentiation and interactions with neurons.

Neurotrophins share high homology in sequence and structure. The neurotrophin genes have arisen through successive duplications of a portion of the genome derived from an ancestral chordate. Their genes share many similarities, including the existence of multiple promoters. The protein product of each gene includes a signal sequence and a prodomain, followed by the mature neurotrophin sequence. Thus, each gene product must be processed by proteolysis to form a mature protein (Richardt et al., 2006).

Nerve Growth Factor (NGF)

NGF has been discovered more than 50 years by Rita Levi-Montalcini and colleagues, as an essential molecule for neuronal survival and axonal growth. It has been well established that neuron survival is affected by modifications in target tissue size. This supported the view that neuron numbers are controlled by the availability of their innervating targets. Since the identification of neurotrophic factors, researchers tried to figure out how neurons die in the absence of trophic support. Garcia et al. showed that the anti-apoptotic protein Bcl-2 is able to rescue sympathetic

neurons from NGF withdrawal induced cell death. Cell death observed upon neurotrophin withdrawal occurs by the intrinsic apoptotic pathway. Bax, a pro-apoptotic member of the Bcl-2 family, is expressed in the developing nervous system and is required for neuron death since neurons from Bax-deficient mice are protected from apoptosis. Moreover, NGF deprivation is associated with Bax redistribution from the cytoplasm to the mitochondria, followed by mitochondria outer membrane permeabilization (MOMP), cytochrome c release and apoptosis. After the release from the mitochondrial intermembrane space, cytochrome c engages the apoptotic protease activating factor-1 (APAF-1) into a heptameric platform called the apoptosome (Ichim et al., 2011).

NGF : structure

The Nerve Growth Factor in its mature form is a homodimeric protein of 26 kDa. Each monomer consists of 120 amino acids in the human form (hNGF) and 118 in the murine molecule (mNGF). The two monomers are joined to one another by weak bonds to form a dimer endowed with the biological function of NGF, in contrast to the monomer that is devoid of such activities. During the 80s through the techniques of genetic engineering was identified the human gene encoding for this molecule. The NGF gene is localized on the short arm of chromosome 1. Neurotrophins are produced as precursors of 260 amino acids with a pre-pro sequence of about 130 amino acids in the N-terminal part which is then removed by a proteolytic cleavage to generate the mature protein.

The pre-pro-neurotrophin is produced in the rough endoplasmic reticulum (RER), and then, thanks to N-glycosylation, is directed to the vesicles of the trans-Golgi where it is cleaved by multiple enzymes. Maturation may also occur in the extracellular space thanks to tPA-plasmin-metalloproteinase. System-tissue plasminogen activator (tPA) is a serine protease abundant in the brain and catalyzes the activation of plasminogen to plasmin. Metaloproteinases, present on the cell membrane or associated to the extracellular matrix, participate to the degradation of NGF. Metalloproteinase-9 (MMP9) is the most important protease involved in this mechanism. MMP-9 is produced as a proforma which requires the proteolytic cleavage of pro-sequence to be activated. Also in this maturation process is regulated by plasmin that, surprisingly, promotes the maturation of NGF but is also able to activate the components involved in its degradation (Bruno et al., 2006).

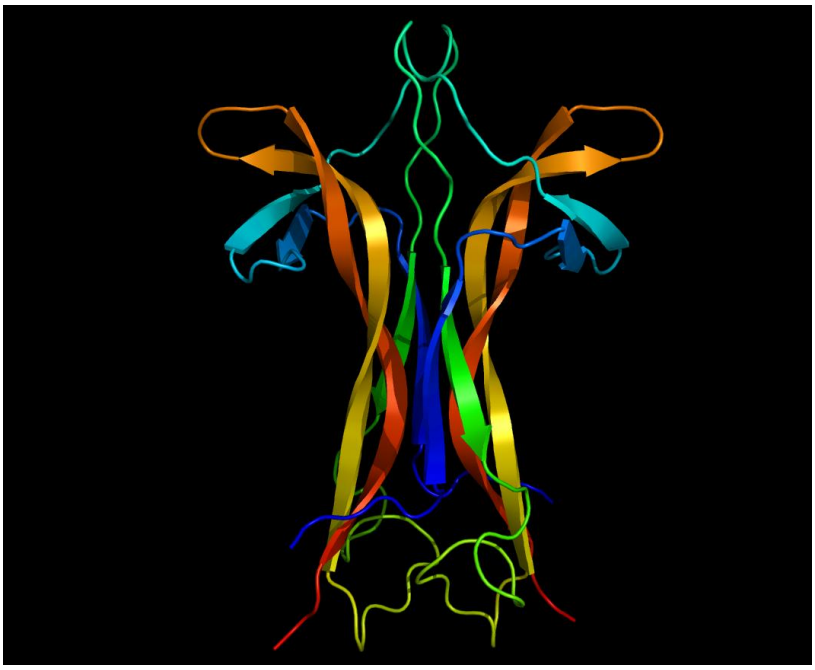


Figure 1.7. Threedimensional structure of NGF

Receptors of Nerve Growth Factor

The neurotrophins interact with two distinct classes of receptors. The first receptor to be discovered, named p75 neurotrophin receptor (p75NTR), was identified as a low-affinity receptor for NGF, but was subsequently shown to bind all neurotrophins with a similar affinity. Moreover, there are different evidence that all pro-neurotrophins can bind and activate this receptor.

In mammals, the three members of the Trk subfamily of receptor tyrosine kinases constitute the second major class of neurotrophin receptors. The four neurotrophins exhibit specificity in their interactions with the three members of this receptor family with NGF activating TrkA, BDNF and NT-4 activating TrkB, and NT-3 activating TrkC.

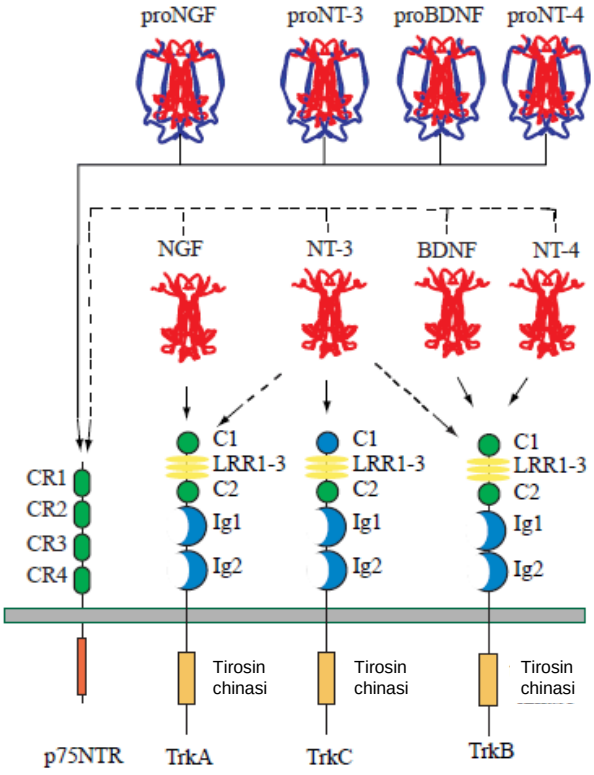


Figure 1.8. Neurotrophins and interaction with their receptors

TrkA Receptor

The TrkA receptor is a transmembrane protein of 140 KDa with tyrosine kinase activity. TrkA is expressed in different areas of the nervous system. In neurons, the pathway regulated by receptor tyrosine kinases regulate survival, differentiation, growth and remodeling of axonal and dendritic, the assembling of the cytoskeleton, trafficking and fusion of the vesicles with the membrane and the formation of synapses (Huang e Reichardt, 2001). Trk receptors contain 10 evolutionarily conserved tyrosines in their cytoplasmic domains, of which three Y670, Y674, and Y675 (human TrkA sequence nomenclature) are present in the autoregulatory loop of the kinase domain that controls tyrosine kinase activity. Phosphorylation of these residues further activates the receptor. Phosphorylation of these tyrosine residues promotes signaling by creating docking sites for adapter proteins containing phosphotyrosine-binding (PTB) or src-homology-2 (SH-2) motifs. These adapter proteins couple Trk receptors to intracellular signaling cascades, which include the Ras/ERK (extracellular signal–regulated kinase) protein kinase pathway, the phosphatidylinositol-3-kinase (PI-3 kinase)/Akt kinase pathway, and phospholipase C (PLC)-γ 1. Activation of Ras is essential for normal differentiation of PC12 cells and neurons. Phosphorylation on Y490 was shown to result in recruitment and phosphorylation of the adapter protein Shc, with binding mediated by the Shc PTB domain.

Shc is then phosphorylated by Trk, resulting in recruitment of the adapter protein Grb-2 and the Ras exchange factor SOS. Activation of Ras by SOS has many downstream consequences, including stimulation of PI-3 kinase, activation of the c-raf/ERK pathway, and stimulation of the p38MAP kinase/MAP kinase-activated protein kinase 2 pathway. Downstream targets of the ERK kinases include the RSK kinases (ribosomal S6 kinase). Both RSK and MAP kinase-activated protein kinase 2 phosphorylate CREB (cAMPregulated enhancer binding protein) and other transcription factors. These transcription factors in turn control expression of many genes known to be regulated by NGF and other neurotrophins.

Activation of phosphatidylinositol-3-kinase (PI-3 kinase) is essential for survival of many populations of neurons. In collaboration with the phosphatidylinositide dependent kinases, phosphatidyl inositides generated by PI-3 kinase activate the protein kinase Akt/protein kinase B. Akt then phosphorylates and controls the biological functions of several proteins important in modulating cell survival. Among the substrates of Akt are BAD, a Bcl-2 family member that promotes apoptosis by binding to Bcl-xL, which in the absence of binding would inhibit the proapoptotic activity of Bax.

Phosphorylation of BAD results in its association with 14-3-3 proteins and prevents it from promoting apoptosis. BAD is also a substrate for MAP kinases, which similarly inactivate its apoptosis-promoting function.

Another demonstrated target of Akt is IkB. Phosphorylation of IkB results in its degradation and activation of NFkB, which is normally sequestered by IkB in the cytoplasm. Transcription activated by nuclear NFkB has been shown to promote neuronal survival.

Phosphorylation of Y785 on TrkA has been shown to recruit PLC-γ 1 directly, which is activated by phosphorylation and then acts to hydrolyze phosphatidyl inositides to generate inositol tris-phosphate and diacylglycerol (DAG). Inositol tris-phosphate induces release of Ca^{2+} stores, increasing levels of cytoplasmic Ca^{2+}. This results in activation of various enzymes regulated by cytoplasmic Ca^{2+}, including Ca^{2+}calmodulin–regulated protein kinases, phosphatases and Ca^{2+}regulated isoforms of protein kinase C. Formation of DAG stimulates the activity of DAG-regulated protein kinase C isoforms. In PC12 cells, protein kinase C (PKC), a DAG-regulated PKC, is activated by NGF and is required for neurite outgrowth and for activation of the ERK pathway. Inhibition of PKC has been shown to inhibit activation of MEK [mitogen-activated protein kinase kinase (MAPKK)/ERK kinase)] but not of c-raf, so PKC appears to act between Raf and MEK in the ERK kinase pathway.

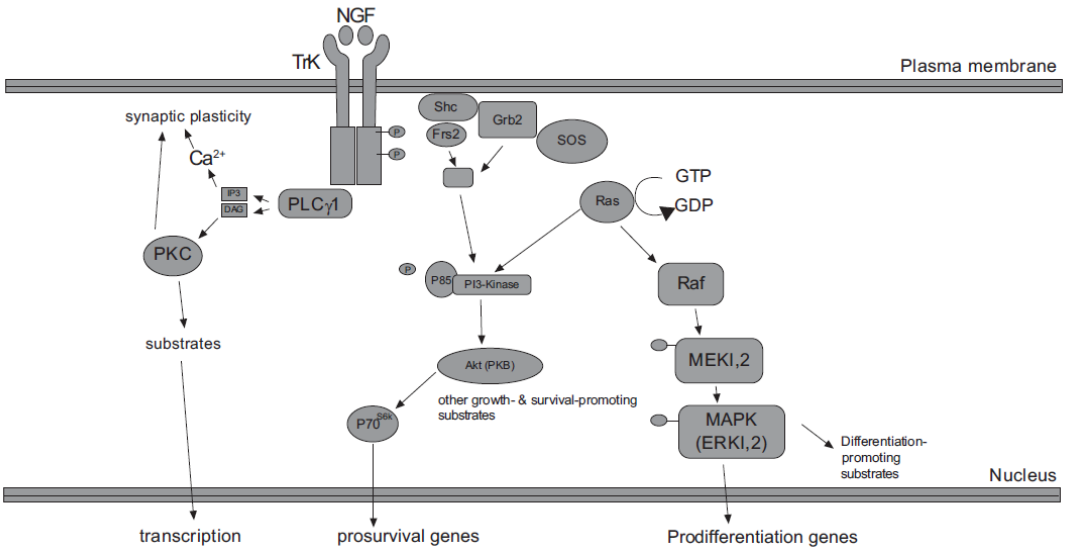


Figure 1.9. Scheme of the signal transduction by TrkA receptor (Škaper et al., 2008).

p75 receptor

The p75 receptor is a transmembrane protein of 75 kDa. The p75 was the first receptor for NTs to be identified. This receptor belongs to the family of TNFR and the cytosolic region contains a death domain that is typical of the members of this family. The expression of this receptor is strongly upregulated under stress, inflammation and neuronal damage, both by neurons and by supporting cells such as astrocytes, microglia and oligodendrocytes in the CNS and Schwann cells in the Peripheral Nervous System (PNS). The p75 does not possess catalytic activity, but is able to recruit various adapter proteins that allow the activation of

multiple signal transduction pathways. Among these adapters: TRAF6 which has activity E3-ubiquitin ligase, NRFI (neurotrophin receptor-interacting factor) that binds the death domain of p75. Through these adapters the binding of p75 by the NTs is mainly reflected in the activation of three pathways: the cascade of Jun kinase (JNK), the activation of NFκB and the way of the ceramide.

-The **JNK** pathway mainly leads to activation of p53, a protein that plays a central role in the regulation of apoptosis. For example, its activation leads to the transcription of bax, which encodes a pro-apoptotic factor. In addition to the activation of p53, another important effect downstream of JNK pathway is the expression of Fas ligand (Fas-L) by neurons, Fas receptor, which promotes apoptosis through another via (Huang and Reichardt., 2001).

-The route that leads to the activation of the transcription factor **NFκB** is the second way of p75 signaling through TRAF6 and activation of the kinase IKK-β (IKB kinase β). This enzyme phosphorylates IKB detaching from NFκB, which is free to translocate to the nucleus where it mediates the transcription of genes for survival.

- **Ceramide** signaling. This molecule is a fatty acid that is produced by acid sphingomyelinase and participates to the regulation of numerous cell signaling pathways activated by p75. It also interferes with the signaling of Trk because it leads to phosphorylation of serine of TrkA and sequesters Raf, thus blocking Erk signaling. Ceramide can also adjust modulate PI-3K in different ways depending on the cell type. For

example, in some cases it inhibits the activation of PI-3K because it blocks the binding with the receptor TrkA through caveolin in lipid rafts (Huang and Reichardt, 2001).

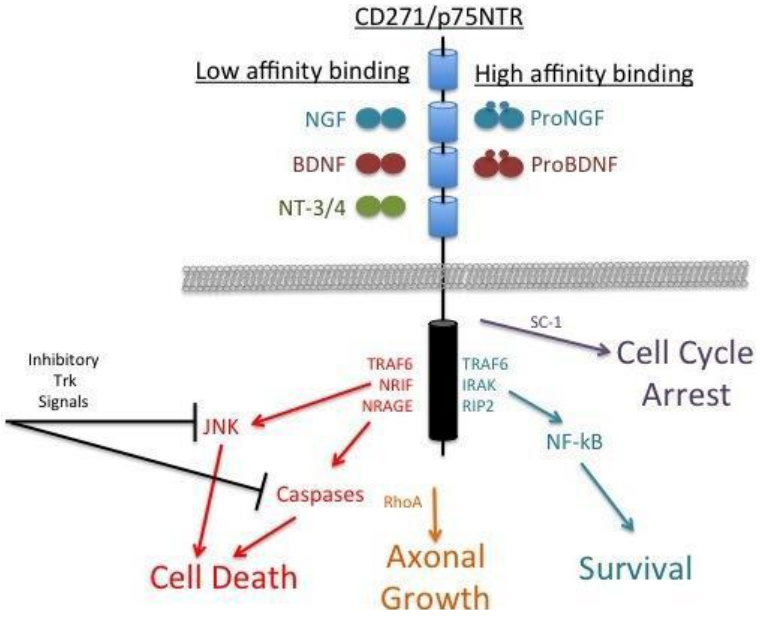


Figure 1.10. Representation of the main signal transduction pathways activated by the binding of neurotrophins and pro-neurotrophin receptor p75 (Ruggeri et al.).

Interaction between p75 and Trk

The p75 was initially considered a coreceptor of Trk. Several studies showed that the co-expression of the two receptors promoted neuronal and glial survival, besides increasing the extension of neuritis (Zaccaro et al.,2001). In an attempt to understand the functions of the mechanisms and processes that result from the activation of the two receptors, have

been clarified roles tend contrasting the pathway triggered. The observation that the two different classes of receptor actually have distinct preferred ligands (proneurotrophins for p75NTR and mature neurotrophins for Trk receptors) provides a partial explanation for this riddle. In any event, the overall picture that emerges from these studies on p75NTR–Trk receptor interactions is that the pro-apoptotic signals of p75NTR are largely suppressed as a result of Trk receptor-mediated signalling and that complex mechanisms have evolved to ensure that ligand engagement of p75NTR functions to promote the efficacy of Trk signalling in conditions where Trk receptors are ligated by neurotrophins. Specificity in signalling through the two families of receptors appears to be regulated through proteolysis of the proneurotrophins (Reichardt et al., 2006).

Interaction between proNGF and p75

The pioneers of this study were Lee et collaborators, who proposed that the cleavage-resistant pro-form of NGF is a high-affinity, functional ligand for the pro-apoptotic p75NTR receptor, whereas the proteolytically cleaved mature NGF is the preferred ligand for TrkA (Lee et al., 2001). The implication of this observation is that the balance between cell survival and cell death could depend upon the ratio of mature and proNGF available to cells expressing TrkA and p75NTR receptors. The discovery of Lee et colleagues brings proteolytic processing to centre stage in the regulation of neurotrophin function. The selectivity of post

secretory proteolytic processing observed suggests that regulation of the synthesis and localization of specific proteases could play a major role in the control of neurotrophin activity. This could be true not only during development, but also in nerve injury and synaptic plasticity responses in the adult. p75NTR expression and function has been associated with a number of disease-states, including stress and inflammation conditions that are also known to regulate the activity of several proteases. Regulation of the expression or activation of specific proteases by neurotrophin signaling could also represent a potent feedback mechanism, to limit or amplify distinct neurotrophin activities (Bruno et al., 2006).

An important issue is the differential ability of proNGF and mature NGF to selectively interact with the p75NTR and trkA receptors. Site-directed mutagenesis and X-ray crystallography studies have shown that the N-terminal domain is crucial for TrkA binding. In the proNGF molecule, however, the presence of the prodomain is likely to impose severe restrictions to the conformation of these residues, preventing them from interacting with TrkA, and thereby hindering activation of TrkA by proNGF (Ichim et al., 2011).

How can the increased affinity of proNGF for p75NTR be explained? This receptor could, in principle, interact directly with a proNGF dimer, the increased affinity reflecting additional contacts with residues in the NGF prodomain. This model, however, might not readily explain the difference in the activation of intracellular signaling pathways by mature and proNGF. An alternative, not mutually exclusive, possibility could be

the oligomerization of proNGF dimers via prodomain interactions (Ibanez CF, 2002).

Further studies have focused on the role of p75 in survival or cell death following the interaction between this receptor and proNGF. These studies indicate that the neurotrophins use three distinct receptor classes. In fact, sortilin was identified as a biologically important neurotrophin receptor that targets the pro domain of proNGF with high affinity. Sortilin is one member of the Vps10p-domain receptor family expressed in the nervous system. Several data suggest that sortilin is a required component for proNGF-dependent death signals via p75NTR. Together with p75NTR, sortilin facilitates the formation of a complex high-affinity binding site for proNGF. Thus, sortilin serves as a co-receptor and molecular switch, enabling neurons expressing Trk and p75NTR to respond to a pro-neurotrophin and to initiate pro-apoptotic rather than pro-survival actions. In the absence of sortilin, regulated activity of extracellular proteases may cleave proNGF to mature NGF, promoting Trk-mediated survival signals. In conclusion, NGF-induced neuronal survival and death depend on an intricate balance between proNGF and mature NGF, as well as on the spatial and temporal expression of three distinct receptors: TrkA, p75NTR and sortilin (Nykiaer et al., 2004).

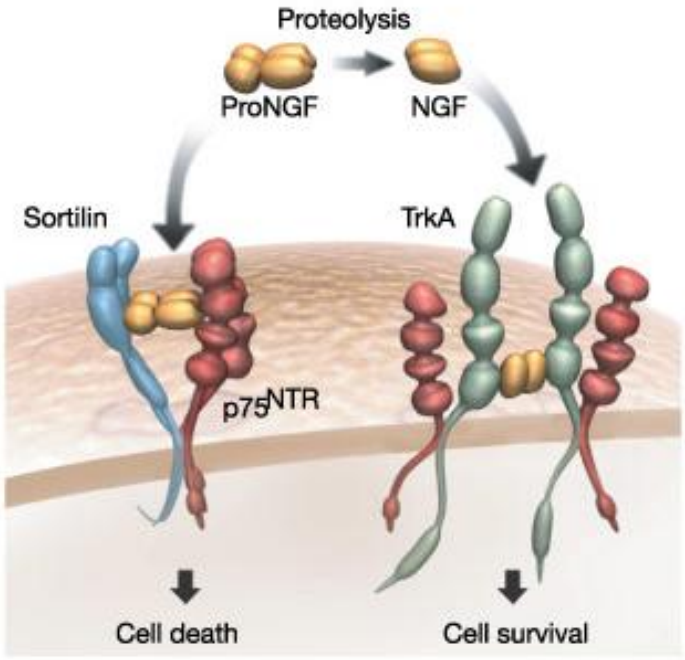


Figure 1.11. Death and survival pathways mediated by p75NTR and TrkA.

Therapeutic approaches based on NGF

Because of its role in regulating neuronal survival, differentiation and maintainance, NGF is an excellent candidate as a therapeutic molecule for several neurodegenerative diseases. However, this important neurotrophic factor, presents two major limits. In fact, the NGF is unable to cross the BBB and has a pro-nociceptive action, since it is able to stimulate the molecular processes that convey signals and sensations of pain. This results in problems at the level of clinical administration: delivery to the brain and the limitations due to side effects, like nociception.

The capacity of NGF to cause pain has been demonstrated in humans in the course of pilot clinical trials in AD patients, as well as during clinical trials to explore the potential use of NGF in peripheral polyneuropathies. This has severely limited, in previous clinical trials, the dosage administrable to patients, jeopardizing the efficacy of the treatment.

The problem of crossing the blood-brain barrier has been overcome by using invasive approaches, such as: 1) neurosurgery for the implant of autologous fibroblasts, engineered to secrete NGF, directly in the brain; 2) the direct stereotactic delivery into the brain of adeno-associated viral vectors encoding human NGF, 3) or the neurosurgical implant into the brain of biopolymer capsules filled with NGF producing cells. Therefore, a safe route for an effective, noninvasive delivery of NGF to the brain is required. NGF intranasal delivery represents an effective approach to meet the required therapeutic window for NGF, leading to NGF accumulation in target brain areas while minimizing its biodistribution to not targeted districts (Cattaneo et al., 2012).

A new interesting strategy for NGF-based therapies has involved the development of small molecules able to exert NGF-like activity. Two approaches have been used to develop small molecules that either exhibit intrinsic neurotrophic activity that retains receptor specificity, affecting only target cells expressing Trk or p75 receptors, acting as agonists or antagonists of these receptors, or that boost neurotrophin synthesis. The advantage of the use of these molecules is their relative stability in vivo; they remain in circulation for more than 24 hours and have excellent

targeting, blood clearance, and bioavailability profiles (Colafrancesco et al., 2011). More recently, however, a valid alternative for neurotrophin. Based therapies is the construction of small molecules that can interact and activate specific receptors. This strategy has been employed to construct small functional mimetics of NGF. This approach might be even more suitable for both pharmaceutical development and therapeutic properties of the molecules. Advantages also include: specific receptor targeting, lower molecular weight, and better pharmacological properties, stability to proteinases, lack of immunogenicity, and lower cost of production.

An example of these peptide molecules is BB14, that behaved as a strong TrkA agonist both in vitro, as demonstrated by its neurotrophic activity on DRG and PC12 cell differentiation through TrkA phosphorylation, and in a rat model of peripheral nerve injury by CCI where BB14 showed to be effective in reducing reactive gliosis and neuropathic behavior (Colangelo et al., 2008 and 2012).

P2X7 Receptors

Given the critical role of microglial activation in neurodegeneration, and the role of purinergic receptors in microglia-mediated neuroinflammatory processes, we have focused our attention on the P2X7 receptors subtype. Evidence from in-vitro and in-vivo studies demonstrate that astrocytes and neurons share receptors and transporters, and glial cells respond to neuronal activity by releasing gliotransmitters, which in turn influence

synaptic signaling. Among these neurotransmitters, ATP plays an important role in neurodegenerative mechanisms. In fact, ATP acts as a neurotransmitter/co-transmitter in the CNS where it has important neuromodulatory and trophic effects. ATP is released from neurons and glia in response to neuronal activity via exocytosis as well as through alternative routes, including hemichannels and other mechanisms. ATP can also accumulate because of release from damaged cells. Convulsive activity produces an overall reduction in brain ATP levels but intense activation of neuronal pathways also triggers ATP release (Skaper et al.). Once released, ATP acts on ionotropic P2X and metabotropic P2Y receptors (that include 7 or 15 subtypes, respectively), and produces a mixture of excitatory and inhibitory effects. The other major class of purinoceptor, P1 receptors, is activated by adenosine. Adenosine is a potent anticonvulsant, and its important contribution to seizure control has recently been reviewed. Now, it is known that P2Y (G-protein coupled) receptors trigger transient intracellular signaling, while P2X receptors trigger sustained signaling, directly linked to chronic neuroinflammation in neurodegenerative processes.

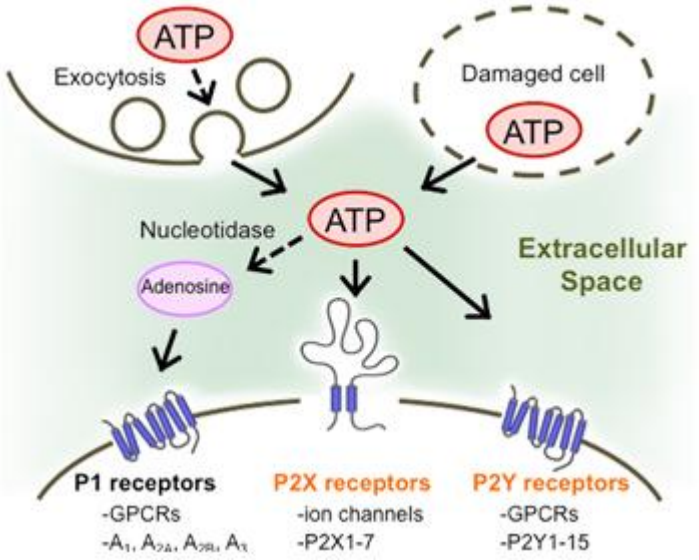


Figure 1.12. ATP and its receptors

P2X7R was initially cloned in 1996 from a rat brain cDNA library and classified as a member of the P2X receptor family due to its amino-acid sequence homology. Similar to other members of the P2X receptor family, P2X7R functions as an ion channel in response to extracellular ATP and is permeable to several small cations such as Ca^{2+}, K^{+}, and Na^{+}. However, distinct from other P2X receptors, P2X7R has a long C-terminal tail that interacts with several proteins and lipids in the cytoplasm and thus ATP can activate multiple intracellular signaling pathways through this receptor. P2X7R possesses some unique properties: (1) much higher concentrations (around the millimolar range) of extracellular ATP are required to activate P2X7R, which is higher than

those required for the other P2X receptors, (2) the channel activity of P2X7R induced by ATP is accompanied by slow or no desensitization during application, while rapid desensitization is observed for other P2X receptors, (3) prolonged activation of P2X7R using higher concentrations of ATP results in pore formation in the plasma membrane leading to the uptake of molecules as large as 900 Da and cell death, and (4) extracellular divalent cations such as Ca^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+} potently inhibit the activation of P2X7R by ATP. Based on these properties, it is believed that ATP exerts specific biological actions through P2X7R activation that never occur when other P2X receptors are activated (Takenouchi et al., 2010).

P2X7R is highly expressed in the cells of monocyte/macrophage lineages and plays important roles in the innate immune system. Although P2X7R is predominantly expressed in microglia, a recent study has shown that this receptor is also expressed in other cell types. Evidence is accumulating for the functional expression of P2X7R in the central and peripheral pre-synaptic termini of neurons, which may be relevant to the modulation of transmitter release, including that of c-aminobutyric acid and glutamate. These results indicate that P2X7R plays important roles in the fundamental regulation of synaptic transmission at the pre-synaptic sites of neurons. P2X7R is also expressed in other cell types, including astrocytes and oligodendrocytes. It is possible that ATP also affects synaptic function by modulation of glutamate release from astrocytes. Taken together, it is possible that impairment of P2X7R expression and

function in both pre-synapse and astrocytes results in the dysregulation of neural transmission and the induction of excitotoxicity correlated with neurodegenerative diseases.

Several evidence have demonstrated that the activation of P2X7R with higher doses of ATP elicits a sustained influx of extracellular Ca^{2+} as well as intracellular K^{+}, but also leads to the activation of multiple intracellular signaling pathways in microglial cells such as the p44/42 ERK kinase, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, AMP-activated protein kinase-a, mammalian target of rapamycin/S6 kinase, NF-kB signaling, and nuclear factor of activated T-cell signaling pathways.

These P2X7R-mediated signals contribute to the expression of various microglia cellular responses induced by ATP.

Through the activation of P2X7R, ATP induces the secretion of pro-inflammatory cytokines and chemokines such as TNFα, and also stimulates the production of superoxide and nitric oxide in microglial cells. These data suggest that P2XRs are a potential target for limiting inflammatory responses, and P2XR antagonists may potentially reduce secondary damage both by directly inhibiting excitatory neuronal damage and by reducing local or systemic inflammatory responses. In particular, in this work we considered the OxATP, a P2X7 receptor antagonist.

Aims of the project

The primary objective of this thesis was to understand the mechanisms of neurodegeneration due reactive gliosis, and to evaluate the possible neuroprotective effects of NGF, based on previous studies showing its "anti-gliosis" activity.

Although neurodegenerative diseases have a multifactorial nature, the scientific literature provides a wealth of information that highlights the important role of reactive gliosis, a process where activated glial cells (astrocytes and microglia) are characterized by overgrowth, hypertrophy and production of cytokines and trophic factors, as a result of any toxic or traumatic insult to the CNS. For this purpose, experiments were carried out in order to define the biochemical mechanisms linked to glial activation and how activated glial can affect neuronal function.

The second objective of this study was to understand the potential mechanism by which NGF is able to prevent this process and restore astroglial and neuronal function. Moreover based on the relevance of mitochondrial dysfunction in neurodegeneration and the role of astrocytes in neuro-metabolic coupling mechanism, we also proposed to evaluate the effect of NGF on mitochondrial function. In particular, it is known that the activation of glial cells could alter the metabolic support provided to the neurons, therefore, we wanted to evaluate the effect of TNFα and NGF on astrocytes bioenergetics.

Finally, another objective of this thesis was the study of the purinergic system in neuroinflammation. In particular, the activation of P2X7R,

induces the secretion of pro-inflammatory cytokines and chemokines such as TNFα and also stimulates the production of superoxide and nitric oxide in microglia cells. These mechanisms are well known in microglia but, less clear is the role of astrocytes. Therefore, we decided to investigate these events in astrocytes to evaluate whether, P2XRs can be considered or a potential target to limit neuroinflammatory process associated with neurodegenerative diseases.

MATERIALS AND METHODS

Primary Cultures

Cortical Neurons

Neonatal (pups of 1-2 days) mice (CD1) are sacrificed by decapitation and the brain is extracted and placed in Dissociation medium with kynurenic acid. After elimination of meninges and olfactory bulb, the neocortex is dissected and placed in a separate dish. Tissues are then minced and enzymatically dissociated by incubation with Trypsin solution (Trypsin and DNase) followed by mechanical dissociation through a fine-polish Pasteur pipette to obtain a homogeneous suspension. Cells are counted and plated at the $15x10^5$ cells/cm^2 (on poly-lysine coated dishes) in Neurobasal medium supplemented with B27, Glutamine, βFGF and Pen/Strep solution. Cultures were maintained at 37°C in 5 % CO^2 and used after 8 days in vitro (DIV).

Cortical Astrocytes

The procedure for preparation of astrocytes and microglial cells is similar to that used for neurons. After dissection tissues are enzymatically and mechanically dissociated in Hank's Balanced salt solution (HBSS). Astrocytes were maintained in 75 cm^2 flasks in Basal Medium Eagle (BME, Sigma) supplemented with 10 % fetal bovine serum (FBS) and antibiotics, at 37°C and 5 % CO^2. Pure cultures (>99 %) of type 1 astrocytes were obtained by shaking flasks at 200 rpm at 37°C to remove type 2 astrocytes, microglial cells, and oligodendrocytes. At confluence, cells were plated onto poly-D-lysine-coated dishes.

In-vitro model of Reactive Gliosis

Astrocytic activation was achieved by treatment with Tumor Necrosis Factor alpha (TNF-α) (10 ng/ml). Mixed cultures of astrocytes and microglia (2%) activated with lipopolysaccharides (LPS,1μg/ml) were also used. The effect of glial activation on neuronal function was evaluated by incubating neurons with conditioned medium (CM) from LPS or TNFα treated glial cells.

Immunocytochemistry

To evaluate the purity of primary cultures (99–99.5 %), cells were plated onto a 12 mm poly-D-lysine-coated coverslip (5000/well). Cells are fixed with paraformaldehyde (PAF) in PBS and then permeabilized in PBS/0,25% Triton for 10 min at room temperature. After blocking of aspecific binding in 10% goat serum/0.2% Tween, coverslips are incubated ON with anti-βIII tubulin (Cell Signaling), rabbit anti-GFAP (1:500, Millipore), and anti-Iba1 (1:50, Abcam) diluted in PBS containing 10% goat serum, followed by washing in PBS, incubation with the appropriate secondary antibody for 1h, counterstained with DAPI (to visualize nuclei) for 1 min, rinsed in PBS and coverslipped with anti-fade mounting medium. Then observed by fluorescence microscopy. Was made the ratio between labeled cells and the total number of nuclei observed. Since this value is the large percentage of specific cells in primary culture.

Astrocytes proliferation

To analyze astrocytes proliferation, cortical astrocytes or mixed glial cells (astrocytes containing about 2 % of microglia) were plated onto 35 mm poly-D-lysine-coated dishes (5000 cells/well). Cells were synchronized by serum starvation for 48h and then switched back to the growth media containing TNFα (10 ng/ml) or LPS (1µg/ml) in the presence or absence of OxATP (100µM). The effect of NGF on proliferation rate was also evaluated by co-treatment of cells with NGF (50 ng/ml). At specific time points (2, 5, 6, 7, 9, 12, and 14 days), cells were detached with trypsin (0.25%) and the number of viable cells was counted by trypan blue exclusion.

Bromodeoxyuridine ELISA Cell Proliferation Assay

Bromodeoxyuridine (BrdU) incorporation analysis was performed by using the BrdU Cell Proliferation Assay (Chemicon) in 96 multiwell plates. Cells were plated onto poly-D-lysine-coated wells (2000 cells/well), synchronized by serum starvation for 48 h and exposed to the growth media containing TNF-α (10ng/ml) or LPS (1µg/ml) in the presence or absence of OxATP (100µM) or NGF (50ng/ml). During the last 24h of treatment, cells are incubated with the 5-bromo-2'-deoxyuridine (BrdU 10µM). When cells are cultured with labeling medium that contains BrdU, this pyrimidine analog is incorporated in place of thymidine into the newly synthesized DNA of proliferating cells. After removing labeling medium, cells are fixed and the DNA is

denatured with fixing/denaturing solution. Then a BrdU mouse mAb is added to detect the incorporated BrdU. Anti-mouse IgG, HRP-linked antibody is then used to recognize the bound detection antibody. HRP substrate TMB is added to develop color and the plates is readed at 450nm. The magnitude of the absorbance is proportional to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation.

Conditioned Medium

When astrocytes and microglial cells were confluent (about 4-5 days) medium was changed with Neurobasal medium supplemented with glutamine, Pen/Strep solution and gentamicin. At the same time cells were treated LPS (1μg/ml) and TNFα (10ng/ml) for 48 hours. Then medium was collected and centrifuged for 10 minutes at 4°C to 6000 rpm to removal death cells and cellular debris. Medium was stored at 80°C and used for following assays.

Cell Viability

Cell survival was analyzed by the methylthiazolyldiphenyltetrazolium bromide (MTT) assay (Sigma). Reduction of the yellow tetrazolium salts (MTT) to the purple formazan is dependent on the activity of mitochondrial dehydrogenases by intact mitochondria and can also be taken as an index of metabolic mitochondrial activity. Cortical neurons or astrocytes (5000 cells/well) were plated in 96 well plates pre-coated with

poly-D-lysine. Following treatments, tetrazolium salts (0.5 mg/ml) were added directly to the culture medium for 4h at 37°C. After incubation, the medium was aspirated and the formazan was dissolved with 100 μl of DMSO (MTT solubilization buffer). The plates were incubated at room temperature, under agitation. The absorbance of samples was measured at a wavelength of 570 nm (700 nm reference wavelength) with a microplate reader. MTT conversion levels were expressed as a percentage of control.

Quantification of ROS

Intracellular production of ROS in cortical neurons and astrocytes was assessed by using the fluorescent probe 2′,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Invitrogen). DCFH-DA is a non-polar molecule that spreads easily within cells and is hydrolyzed by intracellular esterase that remove acetate groups making polar and therefore waterproof. The new polar compound DCFH is oxidized by ROS intracellularly products favoring the formation of 2,7-dichlorofluorescein (DCF), a highly fluorescent molecule that emits at a wavelength of 532nm. DCFH-DA (10μM) was added during the last 30 min of treatments. Cells were then washed with PBS, harvested in 0.25% trypsin, and analyzed by FACS (FACScan, Becton-Dickinson), using the Cell Quest software (BD Bioscience). Flow cytometric measurements were taken with 10.000 cells contained in the gated regions used for calculations. Data analysis was performed with WinMDI software.

Total cell lysates

Cell lysis is the first step to be carried out to study the protein content of a cell. The cells were plated and subsequently treated appropriately. At the end of the treatment the culture medium was aspirated, and were made washing with PBS and TBS. Then, was added to the lysis buffer (20 mMTris pH 8.0; 137 mMNaCl; 1 % Nonidet-P40; 10 % glycerol; 1 mM dithiothreitol (DTT)) containing protease inhibitors (2mM PMSF, 0.1 μg/ml leupeptin, 5 μg/ml aprotinin) and a phosphatase inhibitor cocktail (PhosStop, Roche) in order to prevent protein degradation. The cells were pinch from the surface of the plate, collected in eppendorf tubes and incubated on ice for 20 min. Were, subsequently, centrifuged at 13000 rpm for 15 min to remove cell debris. After centrifugation, the supernatant contained the total protein extracted from the cells; for this reason the latter was collected and protein concentration was determined by Bio-Rad protein assay.

Western Blot Analysis

Once obtained the cell lysates (20–25μg total protein) were dissolved in loading buffer (50 mM Tris, pH 6.8; 2% sodium dodecyl sulfate (SDS); 100mM DTT; 10% glycerol; 0.1% bromophenol blue). Cell lysates were loaded on acrylamide gel to make a electrophoresis analysis. The separated proteins in electrophoresis, to the end of the run, were then transferred to a nitrocellulose membrane by electroblotting. The current transfer proteins from the gel to the nitrocellulose sheet, which is then

saturated with 5% nonfat milk in Tris buffered saline with Tween (TBST) buffer (10mM Tris, pH 7.5; 150mM NaCl; 0.2% Tween 20), blots were probed overnight at 4°C with mouse vGLUT antibody (1:5000; Synaptic System), mouse actin antibody (1:4000), rabbit p75 (1:1000) and rabbit TrkA (1:1000) in TBST, followed by incubation for 1h at RT with HRP-conjugated donkey anti-mouse IgG (1:10,000; Amersham Biotech). Detection was carried out by using the enhanced chemiluminescence system (ECL; Amersham Biosciences). After incubation of the membrane and carried out further washing, the bands were visualized using ECL (solution containing the enzyme substrate is converted into a product that emits chemoluminescenza) and subsequent photographic development in the dark room. The quantification of the bands was obtained using the appropriate NIH ImageJ program.

Protein precipitation with Trichloroacetic Acid (TCA)

Conditioned medium was incubated with trichloroacetic acid (TCA) 6% and triton 0,1% over night to 4°C. TCA is a strong acid able to protonate the carbonyl groups is that the amino groups of proteins. The $NH3^{+}$ provides links to the possibility of trichloroacetate and in this way the hydrophilic amino groups masked so they are no longer able to interact with the solvent and the protein precipitates. Then, the conditioned medium was centrifugated for 15 minute to 4°C to 13000 rpm. Then the pellet was washed 3 times with 70% acetone. The pellet was air dry for 1h and dissolved in lysis buffer.

RNA extraction

Cortical neurons were seeded in plates 60mm polilisinate. Once reached the characteristic conformation and formed the network of synaptic connections, the cells were treated with CM from astrocytes activated by TNFα and NGF for 6h.

For RNA extraction was used a kit Qiagen "RNeasy Mini Kit (50)." The cells were lysed with RLT buffer with the support of a sterile scraper. The lysates were included within the columns provided in the kit, and were performed the steps spin at different speeds and with different buffers, how indicated in the protocol. The RNA was finally eluted by addition of RNase-free water. The RNA was assayed by Bradford assay but using quartz cuvettes. Were then performed readings in a spectrophotometer at 260nm and 280nm. The ratio of the two readings returns the ratio that indicates the purity of the nucleic acid and its value must be between 1.8 and 2. With the readings obtained was finally drawed the concentration of RNA.

Reverse transcription into cDNA

It was used Qiagen kit "RT2 First Strand Kit". Each RNA sample was incubated for 5min at 42°C with a solution of deletion of the DNA containing the buffer GE and RNase-free water. At the end, the solution was kept on ice for 1min. It was then set up the reverse transcription reaction buffer composed of BC3, P2 from the control, from RE3 Reverse Trascriptase Mix and RNase-free water.

Real Time PCR

The analysis of the Real Time PCR was performed using the Qiagen kit "RT2 SYBR Green ROX qPCR Mastermix Synaptic Plasticity" which involves the use of an array with 96 wells, each of which contains the primers necessary for amplifying a single gene involved in synaptic plasticity. The analysis sample was prepared by the addition of SYBR Green Mastermix RT2 and RNase-free water. Were performed: 1 cycle of 10 min at 95°C to activate the Taq polymerase, and 45 cycles of 15sec at 95°C. Was also carried dissociation curve to test the purity of the sample. The data were analyzed by calculating the Ct (cycle threshold: the point of the amplification reaction in which the fluorescence level exceeds a predetermined threshold, above the background signal), the ΔCt (compare the Ct of the sample and the Ct of a reference and therefore of a constitutive gene) and the ΔΔCt (compare the sample of interest with the control sample). Finally, we calculated the fold increase (2-ΔΔCt) which is the parameter that allows the evaluation of the changes of gene expression in different conditions.

Bioenergetic by Seahorse technology

To measure the mitochondrial function of primary cultures of astrocytes was used XF24 Analyzer.

The evaluation with Seahorse physiological state and its alterations in the cells can be obtained by measuring the rate of oxygen consumption (OCR), an indicator of mitochondrial respiration. ATP production can

also take place by means of glycolysis in which the conversion of glucose to lactate is independent of oxygen. The measurement of the lactate produced is done indirectly on the basis of the protons released extracellularly in the medium, providing the value of ECAR (extracellular acidification rate). In particular we used Mitostress test. The cells (25,000) were seeded into 24-well plate in a volume of 100uL of complete medium. The cells were treated with NGF (50ng/ml) and TNFα (10 ng/ml) and at the end of the treatments was replaced the medium of the wells with the specific medium (XF assay medium) for analysis with DMEM not buffered, pyruvate, glucose and glutamine, pH 7.4. The compounds for injection were diluted in DMSO initially and then in XF assay medium. The first compound injected is oligomycin, an inhibitor of the F0 subunit of the ATP synthase. The second compound is FCCP, an ionophore that transports H^+ ions across the mitochondrial membrane, resulting in the collapse of membrane potential. This generates an increase in oxygen consumption and lactate released as trigger compensatory mechanisms. Treatment with FCCP allows to calculate the spare respiratory capacity, breathing capacity decoupled, a parameter proposed as crucial to define the cell fate following physiological conditions ceilings or pathological stimuli. The third injection provides a combination of rotenone and antimycin A, respectively inhibitors of complexes I and III, which will completely block mitochondrial respiration. These compounds were added in each door of the cartridge. After starting the program for running the mitostress test, the instrument

mix the medium in the wells and measure the OCR and ECAR at intervals of 9 minutes. The injection of the first compound occurs after obtaining the first three measurements for the determination of the basal metabolism.

The profile was obtained from the average of the values of each well, and the bioenergetic parameters are calculated directly from the instrument and further analyzed. Normalization was performed by calculating the number of cells for each well using crystal violet.

RESULTS

Realization of an in vitro system of reactive gliosis

The first aim of this study was the elucidation of molecular mechanisms of glial activation on neuro-glial function. To this purpose, we used: an in-vitro model of reactive gliosis based on pure populations of microglial and astrocyte cells treated with Lipopolisaccaride (LPS) or the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNFα), and neuronal cells exposed to conditioned medium (CM) from activated glia. Indeed, the interactions between these three cell types are very important, since synaptic activity requires the participation of astrocytes (tripartite synapse), and neurodegeneration is accompanied by neuroinflammatory processes supported by glial cells (reactive gliosis). First of all it was necessary to obtain primary cultures of astrocytes, microglia and neurons from mice pups and assess the purity by immunohistochemistry. This is a fundamental step for the reliability of the results obtained in the study. To this end, we used antibodies directed against specific markers of neuronal, astrocytic and microglial cells respectively βIII-tubulin, GFAP (Glial fibrillary acidic protein) and Iba1 (Ionized calcium binding adaptor molecule 1). The images in *Figure* 2.1. show examples of cultured neurons (A), astrocytes (B) and microglia (C), which are usually showed a purity of 98-99%.

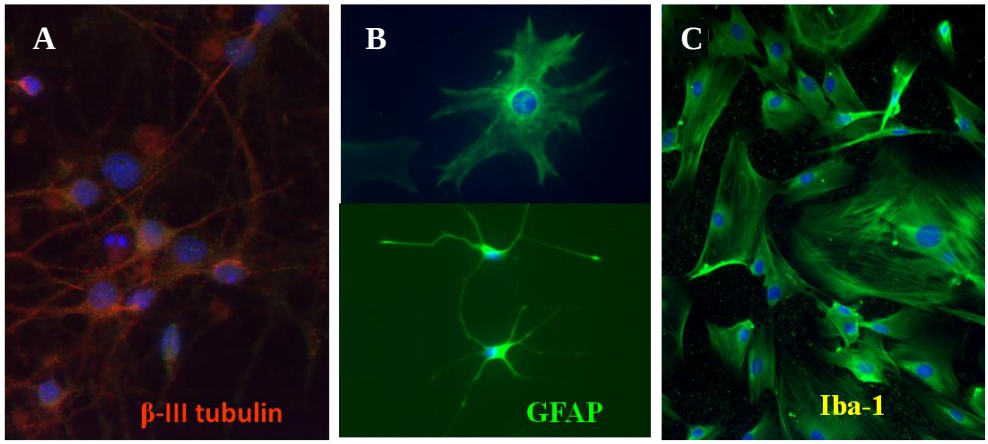


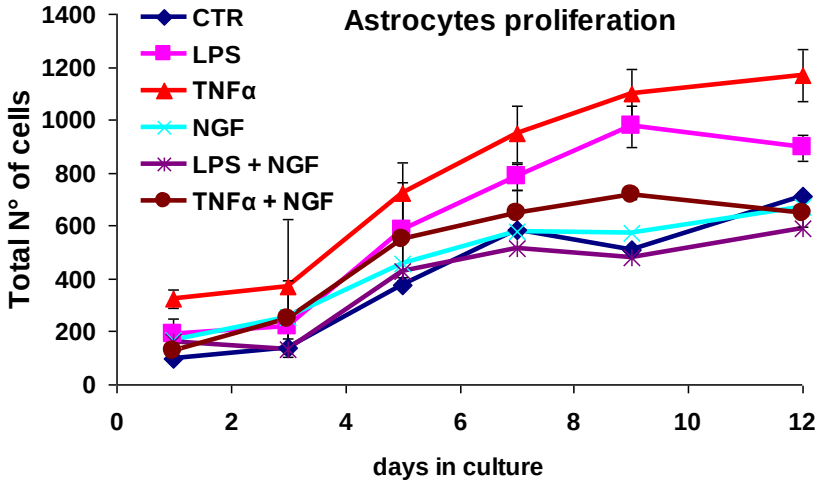
Figure 2.1. Immunohistochemistry images obtained by fluorescence microscopy. **A)** Population of cortical neurons labeled by a primary antibody directed against anti-bIII-tubulin. **B)** Population of cortical astrocytes visualized by using a primary antibody anti-GFAP. **C)** Population microglia labeled with the primary antibody anti-Iba 1.

NGF decreases glial proliferation induced by LPS or TNFα

First, we wanted to assess the effect of glial activation on astrocytic proliferation and the potential anti-gliosis effect of Nerve Growth Factor (NGF). In fact, previous studies of the laboratory (Colangelo et al., 2008; Colangelo et al., 2012) showed an in vivo anti-gliosis activity of this neurotrophin. Moreover, it is known from the literature that are hypertrophy and overgrowth are characteristic markers of reactive gliosis. The glial activation was induced on primary cultures of astrocytes by treatment with Tumor Necrosis Factor (TNFα) or Lipopolysaccharide (LPS). TNFα is one of the main pro-inflammatory cytokines, and its use on an experimental level allows the activation and simulation of

inflammatory processes. This cytokine is actually present in the brain in physiological conditions, but under pathological conditions, it determines important changes of glial cells including the induction of high levels of other pro-inflammatory cytokines (Santello et al., 2012). LPS is, instead, an endotoxin, the main component of the bacterial cell wall of Gram-negative bacteria. LPS acts by activating the immune system through its recognition by Toll-like receptors expressed by cells of the immune system. Stimulation of glial cells by LPS (astrocytes and microglia in particular) causes the production of neuronal pro-inflammatory cytokines, and determines the over-production of free radicals (Dutta et al., 2008). We analyzed glial proliferation following treatment with LPS (1 μg/ml), TNFα (10 ng/ml) and co-treatment with NGF (50 ng/ml) for 2 weeks. Cells were counted at regular time intervals (approximately every 2 days). The results obtained (*Figure* 2.2A) show that, indeed, cell number was dramatically increased during a 14-days time-course by TNFα and LPS treatments. Interestingly, we found that co-treatment with NGF significantly reduced astrocytes proliferation. These data were in part confirmed by experiments of BrdU incorporation in astrocytes treated with TNFα for 72h. The graph in *Figure* 2.2B shows an increase of BrdU incorporation of 1.5-fold after treatment with TNFα, compared to the CTR, and its decrease in samples co-treated with NGF. Taken together these data confirm the anti-gliosis role of NGF.

A



B

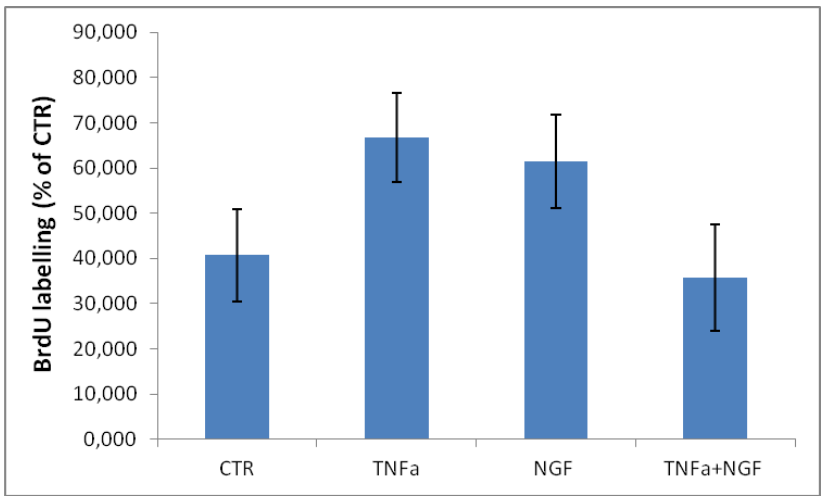


Figure 2.2. Cell proliferation assays. A**)** Growth rate of cortical astrocytes following treatment with LPS, TNFα, NGF and their respective co-treatments. B**)** Rate of BrdU incorporation on astrocytes treated with TNFα in the presence or absence of NGF. Values are expressed as a percentage of control (CTR).

The conditioned medium (CM) from activated astrocytes does not affect neuronal survival

It is now established the important involvement of glia in neurodegenerative processes. Following traumatic or toxic stimuli, glial cells are activated and induce a number of alterations that may be responsible for neuronal death. Astrocytes and microglia are critical cell populations: they are relevant to the trophic support and for the development and maintenance of neuronal networks, therefore their dysfunction can have dramatic conseguences (Miller G., 2005). In order to assess the effects of glial activation on neuronal function, we first performed experiments of cell viability in particular, on neurons treated with conditioned medium from astrocytes and microglia activated by TNFα or LPS.

Signaling pathways activated by TNFα represent a typical marker of the central nervous system diseases. Excessive production of TNFα can lead to cell death of neurons and oligodendrocytes. In fact, TNFα is able to control the release of glutamate by astrocytes, thus regulating synaptic function (Santello et al.,2012).

On the other hand, we performed parallel experiments on neurons treated with CM from LPS-activated astrocytes. This choice was based on the fact that LPS does not act directly on neurons, due to their lack of functional TLRs (Cunningham et al., 2013), thus making possible to specifically assess the effects mediated by glial activation. Primary cultures of pure astrocytes or mixed cultures of astrocytes and microglia

(about 2%) were treated for 48h in complete Neurobasal (specific medium for the cultured neurons) in the presence of TNFα (10 ng/ml) or LPS (10 μg/ml). The culture medium of neurons was then replaced with this CM, while parallel cultures of neurons were exposed to co-treatments or pre-treatments for 24h with NGF (50 ng/ml). After 6h or 24h in the presence of CM (+/- NGF), the MTT assay was performed to assess the viability of neurons. The aim of this experiment was, in fact, to understand the effects (on neuronal viability) of soluble factors released by activated glial cells and the possible neuroprotective effects of NGF. In contrast to our expectation, we observed that treatment with CM for 6 or 24h did not affect neuronal survival, as shown in *Figure* 2.3. Instead, it seemed to increase the viability after 24h. This effect was probably due to the trophic factors (including NGF) produced by astrocytes, especially after inflammatory stimuli. In fact, even the single treatment with NGF increased the neuronal viability because of its neurotrophic properties. Finally, the co-treatment maintained the values of vitality to the levels of control.

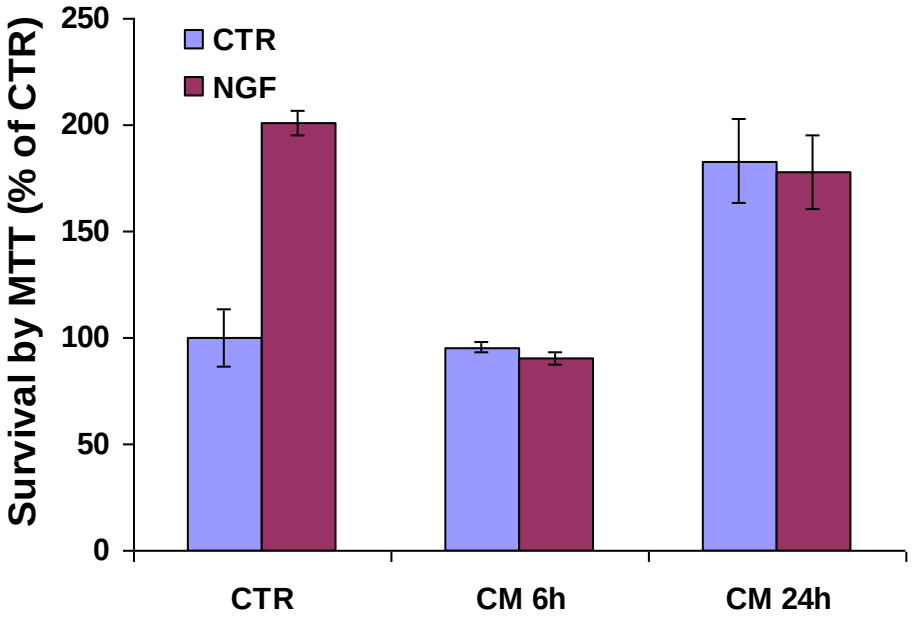


Figure 2.3. MTT assay performed on cortical neurons treated with CM in the presence or absence of NGF for the indicated times. Values, expressed as a percentage of to control (CTR), are the average of 2 experiments with ± DS with 5 samples for each treatment.

CM determines ROS increase in cortical neurons

Although, the treatment of neurons with CM does not appear to affect neuronal viability in a short time (6h and 24h), pro-inflammatory factors released during glial activation in the CM can anyway determine functional alterations that affect neuronal viability at longer times. Indeed, the pro-inflammatory soluble factors produced by activated glial cells may, for example, alter mitochondrial function. Through flow cytometric analysis, we then assessed the concentration of reactive of ROS produced by neurons in response to potential toxic molecules

released by activated glial cells in the CM. ROS are produced by the cells even during the basal metabolism and contribute to regulate normal physiological processes, such as the stimulation of growth factors and activation of the antioxidant defenses. Cells are able to control ROS levels by the use of antioxidant systems. High concentration of ROS, in fact, results in damage to DNA and oxidation of proteins and lipids. However, toxic stimuli linked to neurodegenerative processes determine excessive production of ROS that cause oxidative stress. To assess the role of oxidative stress following glial activation, we analyzed the levels of ROS on neuronal cultures following treatment with CM. Indeed, we found that treatment of neurons with CM from pure population of astrocytes activated with TNFα did not cause a substantial increase in ROS levels compared to the control, unlike the CM obtained from activated astrocytes and microglia. In fact, as shown in *Figure* 2.4, the exposure of neurons for 6h to CM from microglia and astrocytes treated with TNFα showed a significant increase in ROS levels of about 2-3 fold. This is indicative of the importance of microglia in the mechanisms related to the production of ROS. It was also interesting to note that co-treatment with NGF determined a considerable decrease of ROS, although slightly higher than control levels. These data suggest a role of mitochondrial dysfunction in neurodegenerative processes associated with reactive gliosis and an important neuroprotective action by NGF.

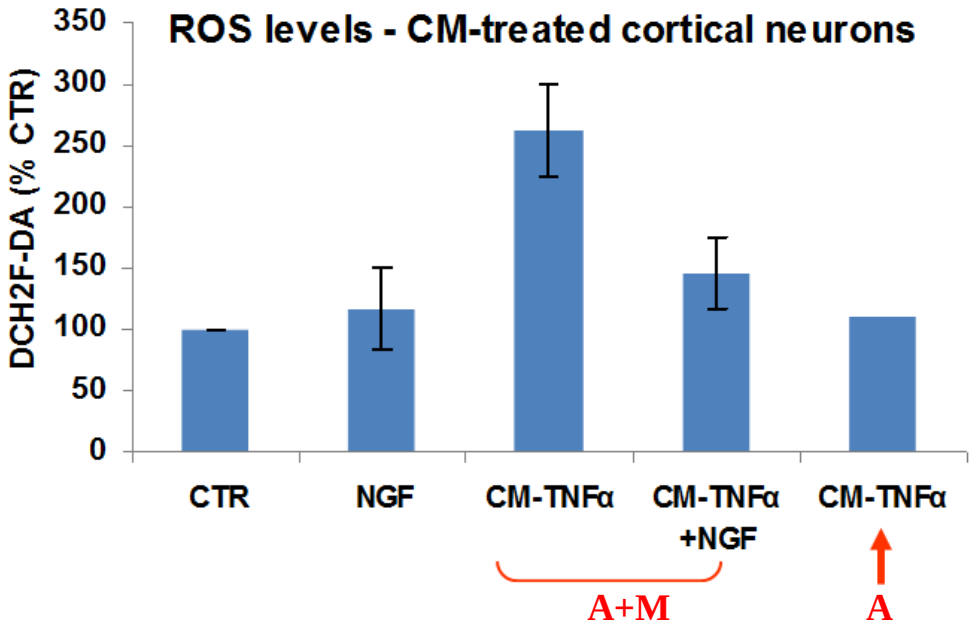


Figure 2.4. ROS levels detected by flow cytometric analysis of DCF-DA fluorescence on neurons exposed for 6h to CM from astrocytes + microglia (A+M) or astrocytes (A) activated with TNFα in the presence or absence of NGF. Values are expressed as percentage of the control (CTR).

CM and NGF determine changes in protein levels of p75 and TrkA receptors

At this point, we decided to investigate the molecular mechanisms that enable soluble factors produced by activated glial cells to act on neuro-glial function in neurodegeneration processes. Considering the neuroprotective role of NGF, we hypothesized that the functional changes observed could be caused by alterations of trophic support. In fact, it is known that astrocytes produce trophic factors, including NGF, and that

various models of neurodegeneration are characterized by an imbalance in the ratio of pro-NGF and NGF. We hypothesized that the pro-NGF, soluble component in the CM, might be responsible for the toxic effects. It is known that induction of cell death can be triggered by signaling pathway related to the interaction between proNGF, p75NTR and sortilin. Alteration of NGF maturation/degradation processes and increase of proNGF levels in the brains of AD patients are currently believed to underlie the vulnerability and atrophy of NGF-dependent cholinergic neurons in AD, as well as in PD and other age-related neurodegenerations (Colangelo et al., 2012). It was also reported that pro-inflammatory cytokines are able to induce neuronal vulnerability by increasing the expression of p75 and sortilin, resulting in a cellular environment more vulnerable to neuronal death induced by proNGF (Choi et al., 2014). We also wondered what could be the mechanism by which NGF exerts its neuroprotective and anti-gliosis action. We hypothesized that this activity could be mediated by receptors: TrkA and p75.

Therefore we first analyzed the levels of receptors TrkA and p75NTR in cortical neurons exposed for 24h to CM from astrocytes activated with TNFα. Parallel cultures were also co-treated with NGF to assess neuroprotection. Receptor levels were quantified by western blot using anti-p75NTR and anti TrkA antibodies.

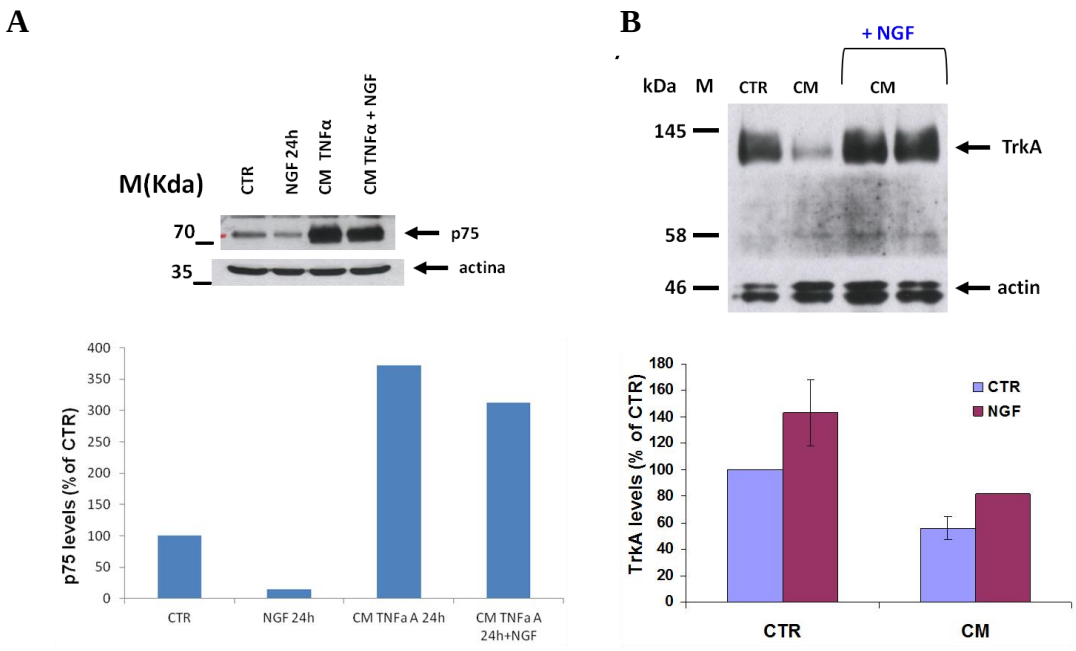


Figure 2.5. Western blot analysis of the levels of p75NTR (A) and TrkA (B) on total cell lysates of cortical neurons treated for 24h with CM from activated astrocytes. At the bottom of each panel are shown the graphs resulting from densitometric analysis of the bands TrkA and p75 normalized to actin. Values are expressed as percentage of the control (CTR).

The results of the Western blot in *Figure* 2.5A show a strong increase in the levels of p75NTR following treatment with CM and their partial reduction in neurons co-treated with NGF. At the same time, TrkA receptor levels were drammatically decreased following treatment with CM. This reduction was completely prevented by the presence of NGF (*Fig*. 2.5B). These data suggest an important role of these receptors in the toxicity mediated by reactive gliosis and neuroprotective action of NGF.

Glial activation determines alteration of MMPs and NGF / proNGF ratio

To better understand the role of pro-neurotrophins, and in particular of pro-NGF, we analyzed the CM from activated astrocytes. The proteins present in the CM were precipitated using trichloroacetic acid (TCA) and analyzed by western blot using an anti-proNGF antibody. Unfortunately western blot analysis shown in *Figure* 2.6 did not allow to identify a single band of proNGF, since more bands of different molecular weights were present. In particular, the bands of 32 and 25 KDa, are known to originate from two alternatively spliced transcripts. Post-translational modifications, including N-glycosylation, also occur in the Golgi network to generate a 40 kDa secretable protein (Al-Shawi et al., 2007) which seems more specifically increased in the CM from astrocytes activated with TNFα. We will need to repeat the analysis with another more specific anti-proNGF antibody.

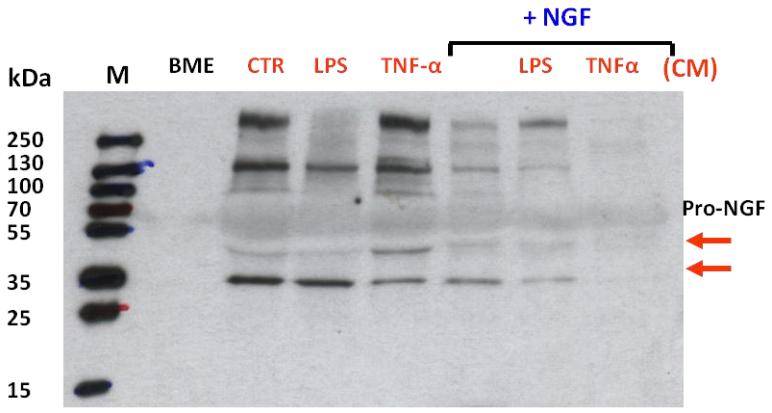


Figure 2.6. Western blot analysis of the levels of proNGF in CM by activated astrocytes. The red arrows indicate the potential bands of proNGF identified. Control is the culture medium from untreated astrocytes.

At the same time, experiments were carried out to examine the possible involvement of metalloproteinases (MMPs). In particular, MMP-9 is known to be primarily responsible for the maturation and degradation of neurotrophins. For this purpose, cultures of astrocytes were treated for 48h with LPS in the presence or absence of NGF and the CM was analyzed by two-dimensional electrophoresis. The analyzes reported in *Figure* 2.7 show a significant increase of MMP-9 in the CM of activated astrocytes, compared to the CTR represented by the culture medium of astrocytes not treated with LPS. This band was completely reduced by co-treatment with NGF. It was also interesting to note that the single NGF treatment reduced the basal activity of MMP-9 in the CTR observed (*Fig.* 2.7). We can assume that glial activation determines the activation of processes that lead to increased degradation of mature NGF by MMP-9, thus causing alteration of trophic support by neurotrophins and

subsequent neuronal degeneration. NGF treatment might favor in part the restore of physiological conditions by inhibiting the activation of enzymes responsible for its degradation.

The model that results from this analysis involves the induction of neuronal cell death mediated by binding of proNGF, present in CM, with p75NTR, whose levels are strongly increased by glial activation.

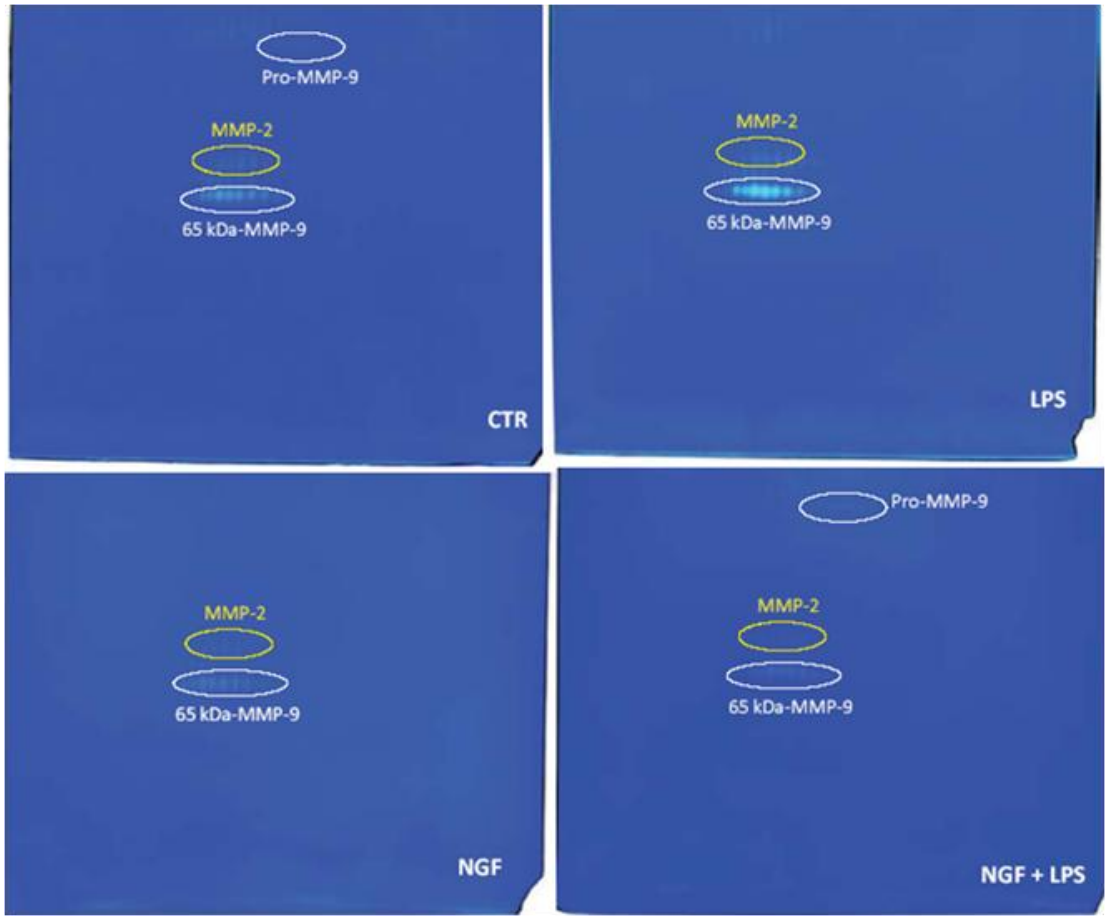


Figure 2.7. Two-dimensional analysis of CM from astrocytes treated with LPS, NGF and co-treatment. The spot associated with MMP-9, MMP-2 and proMMP-9 are indicated.

Altered gene expression following glial activation and NGF neuroprotection

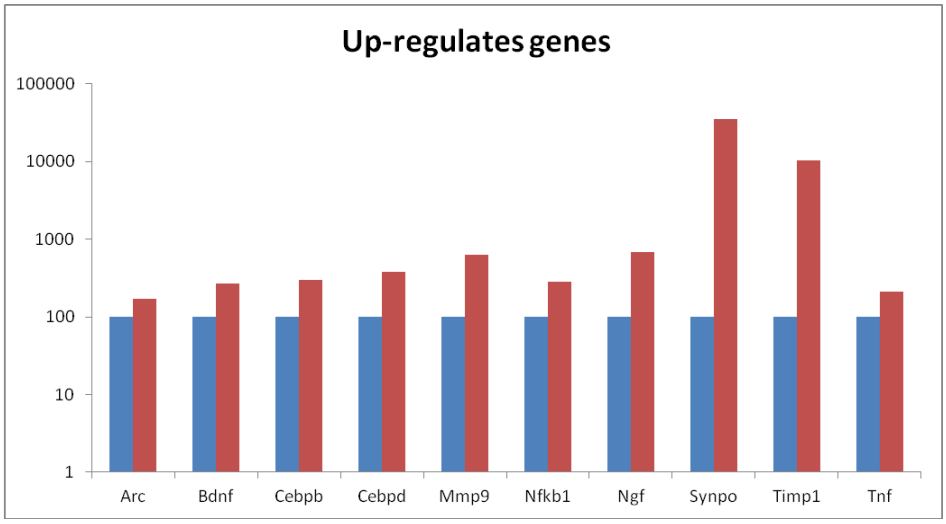
Toxic effects linked to reactive gliosis are related to the induction of several processes that determine morphological and biochemical changes associated with neuronal dysfunction. Based on our data showing the involvement of p75NTR and TrkA receptors in these processes, we decided to investigate whether alterations of the machinery responsible for neurotrophic support (NGF/proNGF ratio and TrkA/p75 receptors levels) correlate with modifications of synaptic function. Of course, modification of function can be due to altered gene expression. In the literature are reported data of transcriptome analisis in activated astrocytes derived from 2 distinct models of reactive gliosis (Zamanian JL et al., 2012). Much less is known about changes in gene expression in neurons exposed to CM from activated astrocytes. Therefore, we attempted to analyze changes of gene expression in cortical neurons in two conditions: the first related to pathological situations (glial activation) relevant to the induction of neurodegenerative processes; the second linked to the neurotrophic effects of NGF. To this purpose, cultures of cortical neurons were exposed for 6h to CM from TNFα-activated astrocytes (*Fig.2.8*A) or treated for 24h with NGF (Fig.2.8B).

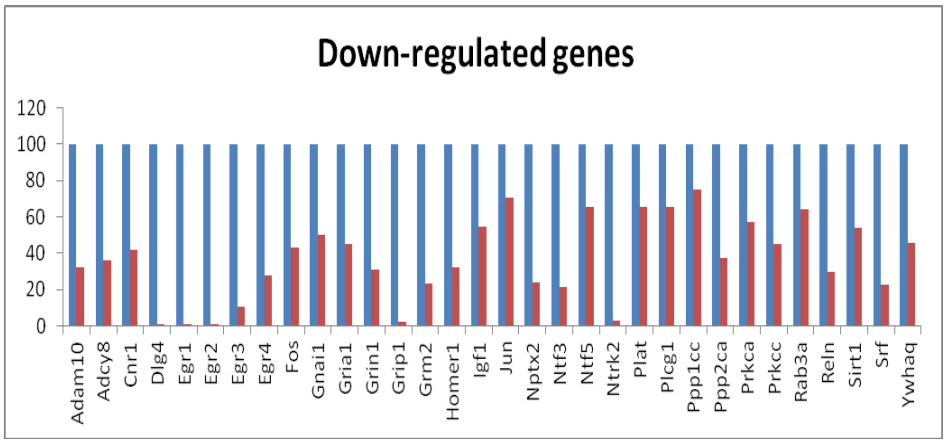
Changes in gene expression were analyzed by Real Time RT-PCR array by analyzing transcript levels for genes involved in synaptic plasticity. The analysis made possible the identification of genes whose expression is upregolated or downregulated in the two experimental conditions.

Many genes were found to be altered and the role of the specific genes will requires further experiments. However, from this first analysis we could identify some functional groups of genes that are up- or down-regulated in a opposite manner in the two experimental conditions. In particular, we found that treatment of neurons with CM-TNFα determined a decrease of most genes involved in Long Term Potentation (LTP) and Long Term Depression (LDP), as well as those present in the post-synaptic density area. These same genes were strongly up-regulated, instead, following treatment of neurons with NGF. Moreover, among the several genes that change in their expression, we observed an increase of NGF and BDNF after reactive gliosis. These variations were, however, accompanied by an increase in the expression, of metalloproteinase-9 (MMP-9), as also observed following glial activation with LPS. These results have allowed us to assume that the CM from activated astrocytes are able to alter the ratio proNGF/NGF and proBDNF/BDNF in favor of the pro-forms by a process mediated from up-regulation of MMP-9 that degrades the mature neurotrophins. Recent studies have also shown that an abnormal expression of MMP-9 is involved in alteration of LTP and, therefore, altered MMP-9 levels might be detrimental for cognitive processes, as observed in some neuropathologies (Wiera et al., 2013). On the other hand, one of the effects of NGF treatment was the downregulation of genes related to NMDA receptors (Grin1, Grin2b, Grin2d, Grip1), which are also involved in the glutamate toxicity.

A

CM 6h


Up-regulates genes
100000
10000
1000
100
10
1
Arc
Bdnf
Cebpb
Cebpd
Mmp9
Nfkb1
Ngf
Synpo
Timp1
Tnf


Down-regulated genes
120
100
80
60
40
20
0
Adam10
Adcy8
Cnr1
Dlg4
Egr1
Egr2
Egr3
Egr4
Fos
Gnai1
Gria1
Grin1
Grip1
Grm2
Homer1
Igf1
Jun
Nptx2
Ntf3
Ntf5
Ntrk2
Plat
Plcg1
Ppp1cc
Ppp2ca
Prkca
Prkcc
Rab3a
Reln
Sirt1
Srf
Ywhaq

NGF 24h

B

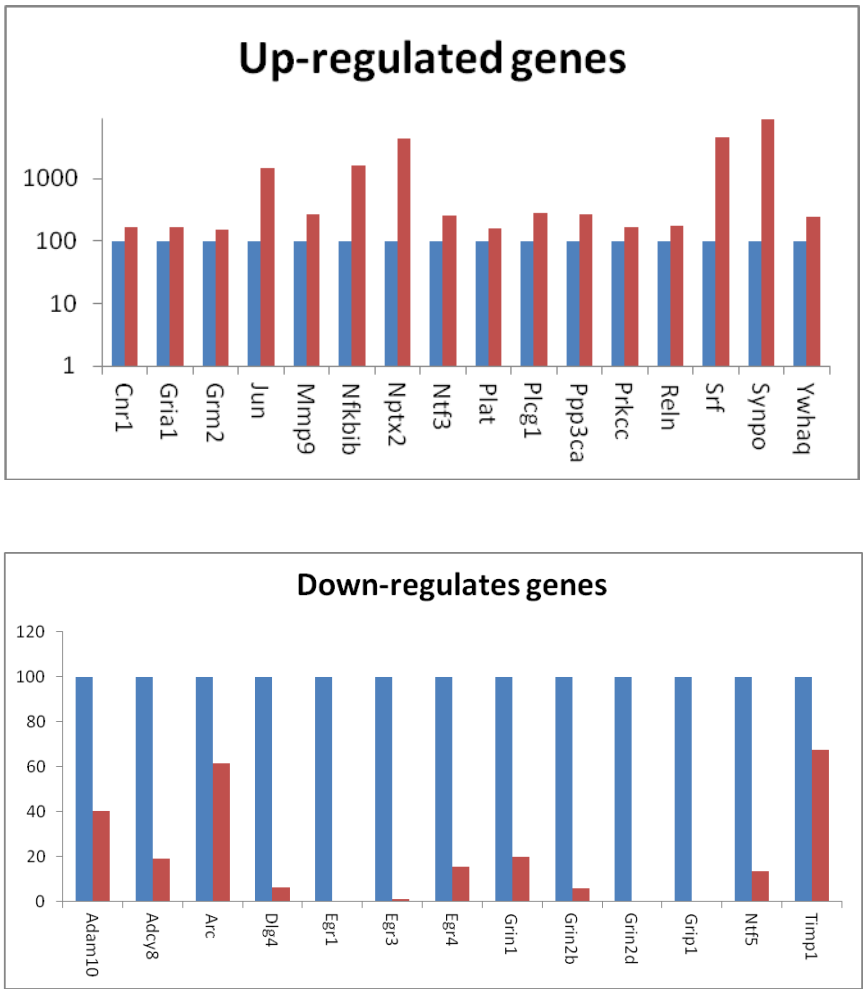


Figure 2.8. Transcriptional analyses performed by real-time RT-PCR. Histograms show the genes that are upregulated and downregulated in cortical neurons treated with CM-TNFα for 6h (A) or with NGF for 24 hours (B)

Mitochondrial disfunction during glial activation

It is well known the key role of mitochondrial dysfunction in many neurodegenerative diseases such as PD or AD, due to oxidative stress triggered for instance by a-synuclein or Amyloid-beta, respectively.

In fact, neurons are especially vulnerable to mitochondrial abnormalities, as they have a high metabolic demand and extraordinary energy requirements. Mitochondrial impairment leads to increased production of ROS and plays a central role in both necrotic and apoptotic cell death (Kim et al., 2010).

Previous studies in our laboratory have shown that deprivation of NGF in PC12 cells (a rat pheochromocytoma cell line originating from the neural crest) results in a strong reduction of mitochondrial membrane potential (Bianco et al., 2011).

On the other hand it is known the role of astrocytes in neurometabolic coupling processes by providing neurons with their metabolic substrates (lactate) in response to increased synaptic activity. Therefore, we assessed mitochondrial bioenergetics on cortical astrocytes by using the seahorse technology. We measured the oxygen consumption rate (OCR) on cultured astrocytes treated with TNFα for 48h in the presence or absence of NGF. Measurements were taken every 9 min, and we scheduled the injection of the inhibitors, (oligomycin, FCCP, and the mixture of rotenone and antimycin A) approximately every 3 measurements. The third OCR measurement provides the value of basal respiration necessary to determine the responses to the various compounds. It is obtained a

profile of mitochondrial respiration, normalized by the number of cells and compared to the fourth measurement (taken as the 100%) in order to better observe the responses to oligomycin and FCCP *(Figure* 2.9).

Preliminary data indicate that TNFα and NGF did not significantly change the response to oligomycin, which gives an indication of how much oxygen is consumed for the production of ATP. Instead, we observed that treatment of astrocytes with NGF determined a change in the response to FCCP, compared to astrocytes under CTR conditions or treated with TNFα. Thus, NGF appears to increase the maximum respiration, an indicator of how a cell can support a metabolic stress that requires considerable amount of energy. The inhibitors of complexes I and III, however, knock down in all cases the consumption of oxygen. NGF can not protect cells against rotenone and antimycin A, probably because of the high concentration used.

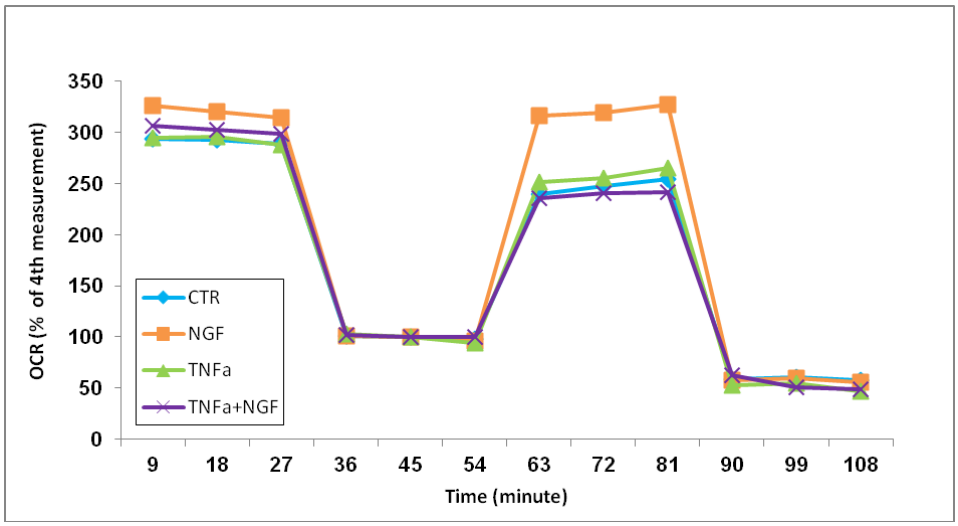


Figure 2.9. Profile of oxygen consumption in astrocytes activated by TNFa in the presence or absence of NGF. Cells (25,000 cells per well) were seeded in 24-multiwell, five wells per condition. Four wells were left without cells to determine the background. TNFa (10 ng/mL) and NGF (50 ng/mL) were added in the medium 48h before the experiment. During the experiment oligomycin (2 mM), FCCP (1 mM)), rotenone and antimycin A (3 mM) were injected at 27, 54 and 90 min, respectively. OCR values were obtained by normalizing by the number of cells contained in the wells and averaging them for treatment. Values are expressed as a percentage of the value of OCR measured at the fourth point of the curve (% of 4th measurement) and are the average of n = 3 experiments.

The maximum value of the three measurements acquired after injection of FCCP (ETC accelerator) is taken as the maximum breathing capacity that cells can sustain. Cells treated with TNFα have a basal respiration substantially lower as compared to astrocytes in control condition. The response to FCCP was significantly lower in the TNFα group compared to NGF, supporting the hypothesis that activation of astrocytes leads to alteration of the astrocytic metabolism, reducing their ability to cope with energy demands that exceed those under physiological conditions.

However, in the literature it is reported that the response to the uncoupler FCCP in other cell types is well above the basal respiration, while in our case, the uncoupler restores only the basal OCR. The hypotheses evaluated in this respect are different: 1) the use of medium with high glucose (25mM) could already push at the maximum the oxidative metabolism of astrocytes, thus the basal respiration is already the maximum possible. We performed an experiment with medium containing low glucose (10mM), but the results were the same (data not shown). The second hypothesis is that primary cultured astrocytes are already activated because of the stress generated by the removal from the tissue. Activated astrocytes could already have an altered metabolism and no further changes could be observed following stimulation with TNFα, while it would be possible to observe a slight improvement due to the administration of NGF.

The seahorse technology also allows to measure the glycolytic metabolism of cultured cells by measuring the ECAR, that is the rate of acidification of the medium by protons dissociated from extruded lactate. During the Mitostress test we also acquired the values of ECAR to provide a first indication of the glycolytic profile of astrocytes activated by TNFα in the presence or absence of NGF (*Figure* 2.10). This is possible because oligomycin and FCCP effectively block the mitochondrial respiration, therefore cells are forced to adopt compensatory mechanisms for the production of ATP, implementing the glycolytic rate.

Also in this case, cells treated with NGF, although not significantly, showed a higher production of lactate compared to astrocytes in the other conditions. It is not clear why astrocytes co-treated with TNFα+NGF show the lower release of lactate.

However, according to these data, it appears that the NGF might affect astrocytic metabolism, although its main role might be at the level of mitochondrial dynamics and biogenesis, which will be the focus of future studies.

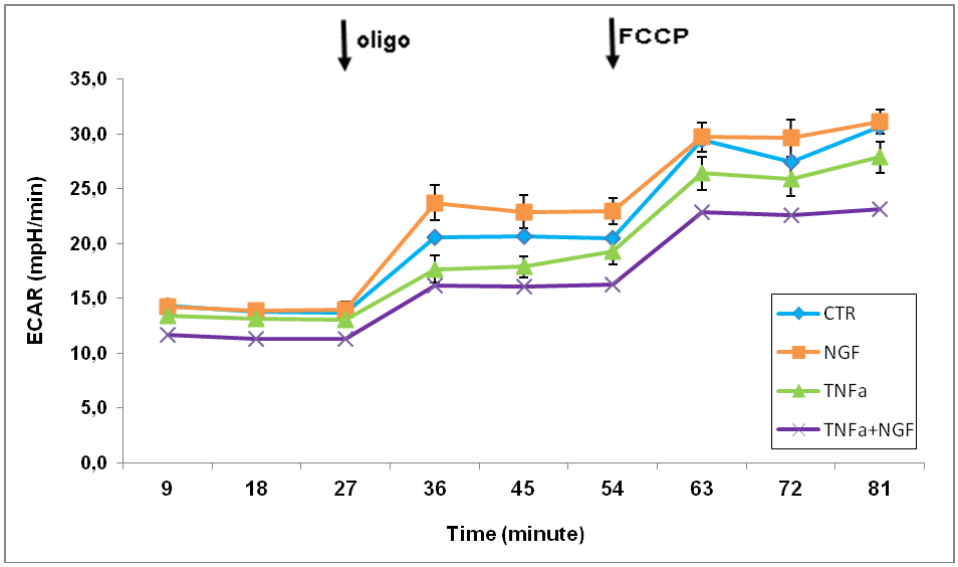


Figure 2.10. Glycolytic profile of activated astrocytes in the presence or absence of NGF. Cells (25,000 cells per well) were seeded in 24-multiweel, five wells per condition. Four wells were left without cells to determine the background. TNFα (10 ng/mL) and NGF (50 ng/mL) were added to the medium 48h before the experiment. During the experiment, oligomycin (2 mM) and FCCP (1 mM), were injected respectively, at 27 and 54 minutes. The values are expressed as variation mpH / min and are the mean ± SEM of n = 3 experiments. The graph shows the error bars of the cells treated with NGF and TNFα.

OxATP decreases glial proliferation induced by LPS or TNFα

It is well known the role of extracellular ATP and purinergic receptors in cytokine regulation and neurological disorders. Starting from these assumptions, another objective of this thesis was to investigate the role of the purinergic system in neuroinflammation. Also, several evidence from in-vitro and in-vivo studies demonstrate that astrocytes and neurons share receptors and transporters, and glial cells respond to neuronal activity by releasing gliotransmitters, which in turn influence synaptic signaling. Among these receptors, we focused on purinergic P2X7 receptors that are expressed on both pre and post-synaptic neurons, as well as on microglia, astrocytes and oligodendrocytes. A number of reports have shown that the activation of P2X7R induces the secretion of pro-inflammatory cytokines and chemokines, such as, TNFα and also stimulates the production of superoxide and nitric oxide in microglia cells. These mechanisms are well known in microglia but, less clear is the role of astrocytes. The P2X7 receptor may thus represent a critical communication link between the nervous and immune systems, while providing a target for therapeutic exploitation. In particular, to study the effect of purinergic system on astrocytes activation we used Oxidized ATP (OxATP), that is an antagonist of P2X7 receptors.

We first analyzed glia proliferation following treatment with LPS and TNFα for 2 weeks. Cells were first synchronized by serum starvation for 48h and then switched back to the growth media containing TNF-α (10

ng/ml) or LPS (1μg/ml) in the presence or absence of OxATP (100 μM). The cell number was dramatically increased during a 14-day time course both in astrocytes cultured in the presence of TNFα (10 ng/ml) and, to a lesser extent, in LPS-treated glial cultures. Interestingly, we found that chronic co-treatment with OxATP (100μM) significantly prevented astrocyte proliferation induced by TNFα (*Figure* 2.11A) as well as the growth of mixed glial cells treated with LPS (1μg/ml), as compared to their corresponding treatments with TNFα or LPS alone. The effect of OxATP on glial proliferation was further investigated in the presence of BrdU during a 10-day time course. As shown in *Figure* 2.11B, chronic co-treatment with OxATP (100 μM) determined a strong reduction of BrdU istaining, thus confirming the data described above. These data indicate that the decrease of cell number elicited by OxATP was not the consequence of decreased survival.

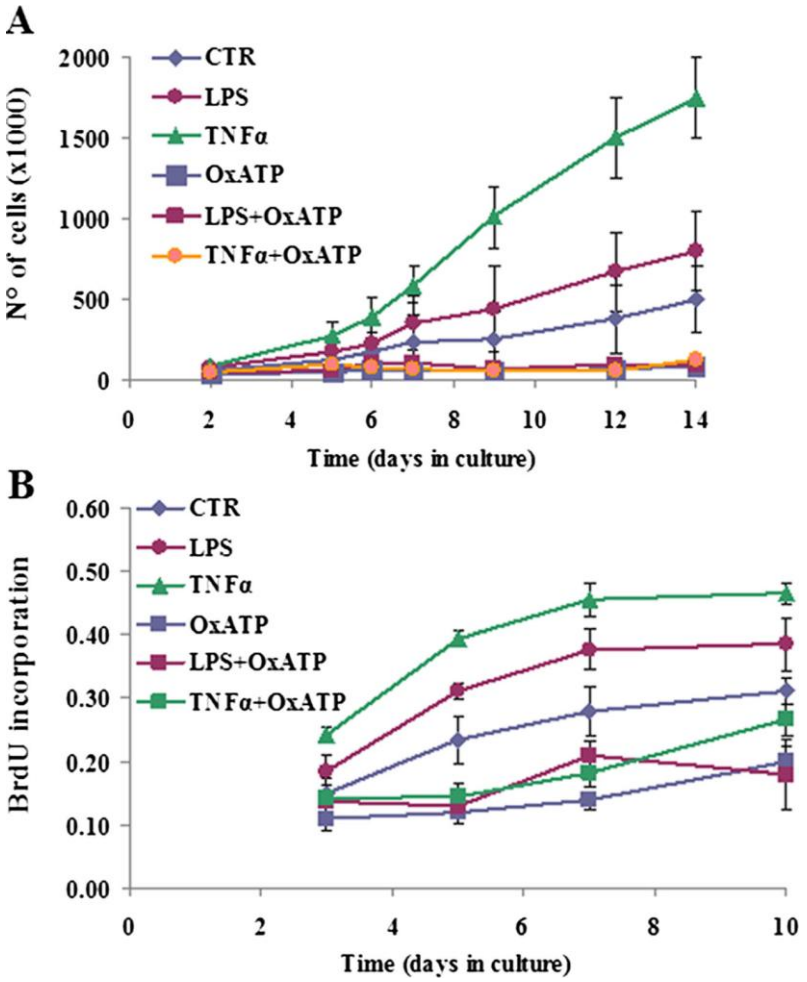


Figure. 2.11. Reduction of astrocyte proliferation by OxATP. A) Cell count of astrocytes or mixed glial cells treated with TNF-α (10 ng/ml) or LPS (1 μg/ml), respectively, in the presence or absence of OxATP (100 μg/ml) for the indicated times. Data are the mean±SEM of five independent experiments. B) Proliferation rate of astrocytes or mixed glial cells stimulated with TNF-α (10 ng/ml) or LPS (1 μg/ml), respectively, in the presence/absence of OxATP (100 μg/ml) for the indicated times. BrdU (10 μM) was added during the last 24 h of treatments. Data are the mean±SEM of three independent experiments, each performed with four to six samples for each treatment.

Effect of OxATP on neuronal and astrocytic viability

In addition we evaluated astrocytic and neuronal viability by the MTT assay. As shown in the graph (*Figure* 2.12A), we found that treatment with OxATP (100 µM) up to 72h did not change the survival of cortical astrocytes, which instead was strongly decreased by exposure (ON) to hydrogen peroxide (H_2O_2 200 µM), used as a positive control. It was also important to note that OxATP did not affect the survival of cortical neurons, in contrast to the well-known toxic effect of glutamate, which led to a 50% reduction of neuronal viability after 16 hr treatments.

In addition, flow cytometric analysis using dichlorofluorescein diacetate (DCF-DA) staining revealed that treatment of cortical neurons with OxATP for 6h determined a 40% reduction of basal levels of ROS (*Figure* 2.12B).

A similar effect was also found in astrocytes: FACS fluorescence profiles in *Figure* 2.12C show that OxATP was able to decrease both basal ROS levels and prevent ROS production induced by a 6-hr exposure of astrocytes to H_2O_2 (Figure C-D).

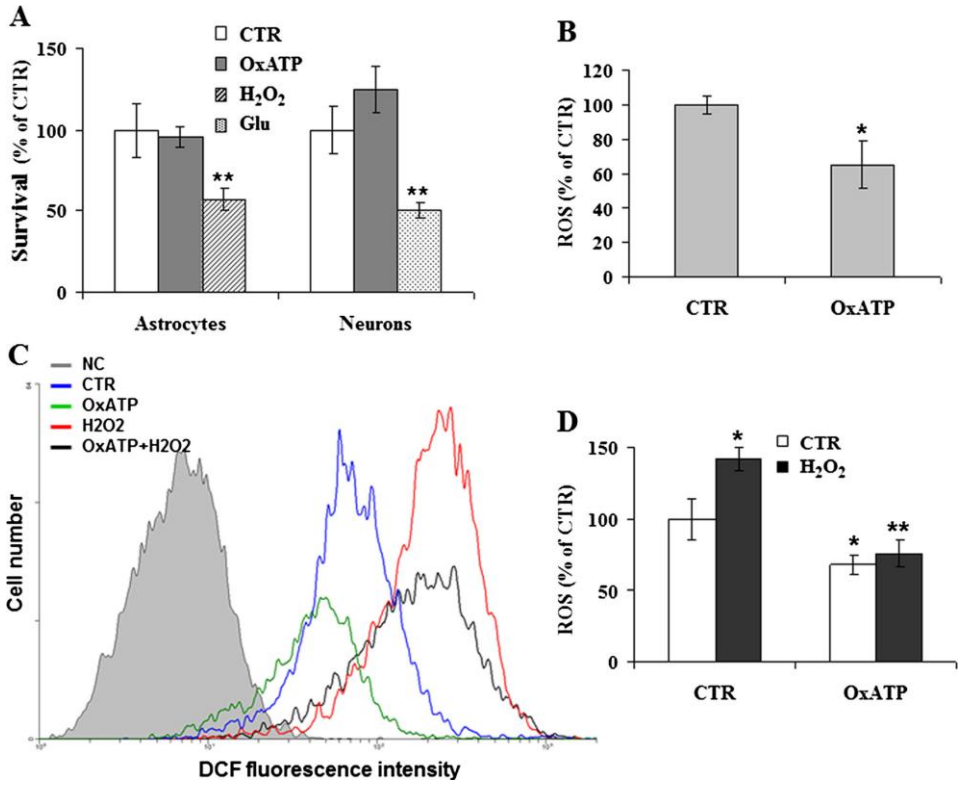


Figure. 2.12. Effect of OxATP on neuronal and astrocytic viability. A) Survival by MTT assay of cortical neurons and astrocytes following treatment with OxATP (100 μg/ml) for 72 h. Treatments with H2O2 (200 μM, dashed bar) or glutamate (200 μM, dotted bar) for 16 h were used as positive controls for astrocytes and neurons, respectively. Data, expressed as a percentage of control (CTR), are the mean±SEM of three independent experiments, each performed with four to six samples for each treatment. B–D Measurement of ROS levels in neurons (B) and astrocytes (D) under basal conditions or following treatment with OxATP for 6 h. As a positive control, astrocytes were treated with H_2O_2 (100 μM) for 6 h. Cells were loaded with DCFH-DA (10 μM) during the last 30 min of treatments and analyzed by FACS. Flow cytometric measurements were taken on 10,000 cells. Data, expressed as a percentage of CTR, are the mean±SEM of three experiments in duplicate (*p≤0.05; **p≤0.001, versus their respective CTR; ANOVA and Dunnett's test). c Representative FACS fluorescence profiles of astrocytes treated with H2O2 (100 μM) in the presence or absence of OxATP (100 μM).

OxATP pre-treatment restores levels of vGLUT expression

Several studies have demonstrated the important role of astrocytes in regulating glutamate, the most important and abundant excitatory neurotransmitter with an important role in neuronal activation (Abazyan et al., 2014). In addition, the involvement of TNF in regulating glutamate release from astrocytes during physiological conditions has been found in TNF - and TNF receptor 1 knockout mice, pointing to a permissive role for the cytokine in the exocytosis of glutamate from astrocytes (Cali et al., 2014). Based on this evidence, we wanted to analyze the expression of v GLUT, the trasporters of glutamate, because of its role in excitotoxicity, in response to purinergic inhibition. WB analysis in *Figure* 2.13A shows that vGLUT levels were dramatically reduced in cortical neurons exposed for 24h to the conditioned medium (CM) from astrocytes activated by TNFα (10 ng/ml) or LPS (1 μg/ml) for 48h. These modifications were not observed when neurons were preincubated for 2 h with OxATP (100 μM) before addition of CM-TNFα or CM-LPS (*Figure* 2.13A–C). A decrease of vGLUT expression was also observed in TNFα-activated astrocytes for 72h (*Fig*. 2.13B) as well as in mixed glia treated with LPS for 72h. Interestingly, under all experimental conditions, the reduction of vGLUT levels was reversed by co-treatment with OxATP (100μM) (*Fig.* 2.13B–D). These data clearly

suggest that the purinergic system, mainly through P2XRs, modulates the expression of glial and neuronal glutamate transporter vGLUT.

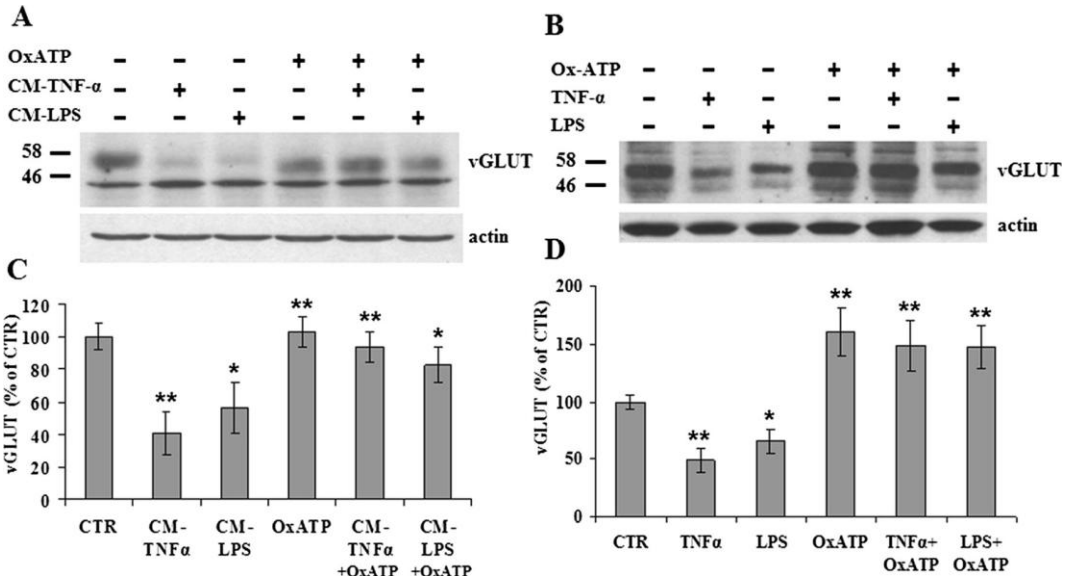


Figure 2.13. Expression levels of vGLUT in cortical neurons and astrocytes. A) Representative Western blot (WB) of cortical neurons treated with CM from TNF-α (10 ng/ml)-activated astrocytes (CM-TNF-α) or CM from mixed glial cells treated with LPS (1 μg/ml) (CM-LPS) in the presence or absence of OxATP (100 μg/ml). B) Representative WB of cortical astrocytes or mixed glial cells activated by TNF-α (10 ng/ml) or LPS (1 μg/ml), respectively, with orwithout OxATP (100 μg/ml). Total lysates were processed by WB using anti-vGLUT antibody. Blots were probed with β-actin to normalize for protein content. C), D) Quantification of bands was achieved by NIH ImageJ software, and protein levels, normalized by the actin content, were expressed as a percentage of control (CTR)±SEM (*p≤0.05; **p≤0.001, versus their respective CTR; ANOVA and Dunnett's test)

DISCUSSION

The structure and functions of the nervous system are defined by the interactions between the cells that constitute a dense network of interconnections. Each cell population plays important roles for the maintenance and proper functioning of the system. Neurons represent the cellular units essential for the transmission of information, that takes place by means of the propagation of electrical impulses. Although glia was considered simply as the cellular components for structural and trophic support, numerous studies have shed light on their relevance in brain homeostasis and function. Glial cells promote the velocity of conduction of nerve impulses (oligodendrocytes), represent the immune system of the nervous system (microglia), and engage on trophic support and metabolic influence of synaptic plasticity, thus regulating brain homeostasis (astrocytes).

More recently, glial cells are belived to be the main actors in neurodegeneration due to their activation of inflammatory processes called reactive gliosis. Microglial cells, represent the macrophages of the central nervous system. Toxic impulses and harmful stimuli determine microglial activation that results in cell migration to the site of damage, and in many morphological and functional changes. The activated microglia then triggers important processes that aim to restore homeostasis and activate inflammatory processes. The microglia, in these conditions, produces pro-inflammatory cytokines and soluble mediators of inflammation that are able to act on cells by determining astrocyte activation. This results in morphological changes, such as hypertrophy

and overgrowth, as well as in the production of pro-inflammatory cytokines and exacerbation of the inflammation. It is known the dual identity of the reactive gliosis that is activated in an attempt to restore a balance of the system, but is resolved in most cases, in the promotion of neurotoxic processes. The astrocyte dysfunction observed in these processes may favor the neuronal degeneration through a plurality of processes.

A characteristic of glial cells is the production of neurotrophins, trophic factors that promote the preservation, development and survival of neurons. Of this class of proteins, NGF was the first to be discovered is the NGF. The studies of this thesis project have been able to clarify processes related glial activation and the activity of NGF as a neuroprotective factor. We tested the ability of this molecule to reduce the reactive gliosis induced by TNFα and LPS, using the hyperproliferation as a parameter for the evaluation glial activation. TNFα, the most known pro-inflammatory cytokine, has been used in vitro to induce activation of glial cells, making them able to produce, in turn, high levels of pro-inflammatory cytokines. LPS was used, instead, as an inducer of neuroinflammation, for its ability to act on Toll-like receptors present on astrocytes and microglia, but not on neurons. Since LPS can not act diretly on neurons, the use of LPS to stimulate glial activation, made it possible to have a “more” controlled system to specifically analize only the effect of glial activation on neuronal function.

To understand the neuroprotective mechanisms induced by NGF is necessary to understand the processes related to it. The functions of this neurotrophic molecule are exerted through interaction with two different types of receptors. In fact, TrkA interacts with the mature form of NGF and induces signal pathways that promote neuronal survival. Instead, the preferential interaction of pro-neurotrophins (pro-NGF) with p75 is known to activate the pathways that lead to neuronal apoptosis or decreased glial proliferation. Thus, the key to these processes may reside in different specific receptors for the mature and immature forms of NGF, as well as the balance of proNGF and mature NGF regulated by their maturation/degradation by tPA/plasmin and MMPs system.

One of the objectives of this thesis was to investigate whether the receptors for NGF are involved in neurotoxic processes. In fact, soluble factors present in the CM from astrocytes and activated microglia are able to determine the increase of ROS. The production of ROS is a typical signal of degenerative processes as the neurons are extremely vulnerable to oxidative stress. Numerous studies have shown a correlation between increased production of ROS and mitochondrial dysfunction in neurodegenerative diseases. The mitochondrion is, in fact, also involved in the processes that regulate cellular apoptosis. The increase in outer membrane permeability induced by toxic stimuli promotes the release of cytochrome c into the cytoplasm, which is able to activate the proapoptotic processes (Zuo et al., 2013). The role of ROS produced by dysfunctional mitochondria is well documented. For example, Yan and

colleagues showed the strong correlation between mitochondrial dysfunction and the increase of ROS in Parkinson. In this case, the degeneration of dopaminergic neurons was led by mitochondrial pathogenic mechanisms, which resulted in the release of ROS (Yan et al., 2012).

NGF might influence these mechanisms by partially restoring normal physiological processes. The assessment of ROS production is not sufficient to substantiate a primary key role of mitochondria in these inflammatory processes; mitochondrial dysfunction may in fact be a secondary consequence of the other important processes upstream. Reactive gliosis and NGF may act, in fact, on neurotoxic processes that do not directly involve the mitochondria but can create a toxic environment that finally results in mitochondrial dysfunction.

On the basis of these knowledge, we performed experiments to define the molecular changes in neurons mediated by activated glia or following NGF treatment. In fact, astrocytes and neurons, at the physiological level, are closely linked by an interchange of substrates that reciprocally support their metabolism. Neurons have a mainly oxidative metabolism: the lactate supplied by astrocytes is taken up by neurons, enters the TCA and provides electrons for oxidative phosphorylation. Astrocytes, on the contrary, have a more glycolytic metabolism, so the ATP produced and the reducing power come respectively from glycolysis and the pentose phosphate pathway. The glucose in the circulation is internalized by astrocytes and converted to lactate, then released into the extracellular

space and made available to neurons according to the model of Astrocyte Neuron Lactate Shuttle (ANLS) (Belanger, 2011). If the metabolism of astrocytes is altered, they would lose the capability to support neuronal metabolic activity. Therefore, we investigated whether glial activation by TNFα and/or treatment with NGF determined a change in the metabolic profile of astrocytes. With the technology Seahorse we could measure in real time the oxygen consumption and lactate production of cultured astrocytes, which are indicative, respectively of the trend of the glycolytic and oxidative metabolism of cultured cells.

From our preliminary experiments, it seemed that TNFα reduced both the maximum breathing capacity and the production of lactate compared to the control, while the NGF improved both parameters, indicating that this neurotrophin results in an overall improvement of cell metabolism. Therefore, administration of NGF could result in an improvement of the resistance of astrocytes to stresses that require greater amounts of energy and an increase of lactate production in support of neuronal activity. Unfortunately we could not observe significant differences between astrocytes activated by TNFα and astrocytes co-treated with NGF. This issue will be further investigated in future studies. However, at the same time, using biochemical approaches, we were able to identify a key component that may participate in the mechanisms of neurotoxicity induced by glial activation. The component in the conditioned medium to determine these effects could be the proNGF by activation of the apoptotic pathway associated with p75NTR; in fact, levels of this receptor

increase as a result of glial activation induced by TNFα or LPS. On the other hand, glial activation determined a decrease of TrkA, that was prevented by co-treatment with NGF. Thus, differential reduction of p75 (during glial activation) or TrkA (by NGF) would favor the activation of two different patway with two opposite consequences.

To better define the mediators and the components involved in these pathways we performed RT-PCR analysis. Our experiments have shown changes in the expression of the majority of genes involved in synaptic plasticity. Functional gene grouping showed that major changes were presents in groups of genes relevant to LTP, LDP and post-synaptic density area. Moreover, it was important to note an increase in NGF and BDNF mediated by reactive gliosis, as well as the simultaneous increase of MMP-9 which allowed us to hypothesize a neurotoxic mechanisms that would involve also lead to increase degradation of mature neurotrophins and alteration of proNGF/NGF ratio. In fact, two-dimensional analysis of CM from astrocytes activated by LPS also revealed an increase of MMP-9, reduced by co-treatment with NGF. The single treatment with NGF also showed a decrease of MMP-9 relative to the control. This latter finding allows us to suppose mechanisms by which NGF is able to inhibit its degradation. In fact, these changes appear to show the operation of processes that favor the degradation of mature forms to determine an imbalance favorable to the pro-forms, possible factors responsible for the induction of neurodegenerative mechanisms. It will be necessary to conduct further experiments to confirm these data and it could be

interesting to identify the elements that determine the switch from apoptosis to survival. Although we have hypothesized that the increase in proNGF would be a key process in the alteration of neuronal function, we were unable to verify and demonstrate the increase in the pro-form in association with glial activation. Further studies will be necessary to measure levels of proNGF (or pro-BDNF) as soluble factor released by activated glia and prove their relevance to neuronal dysfunction. Although neurodegenerative diseases are multifactorial diseases, reactive gliosis appears to be common to various diseases of the nervous system: Alzheimer's disease, Parkinson's disease, Huntington's disease and so on. The anti-gliosis activity of NGF revealed by these studies, as well as by previous in-vivo studies of my lab, would be relevant for the development of NGF-based therapies for several neuroinflammatory diseases. To this end, it will be essential to evaluate also the anti-gliosis activity of the NGF-like peptide, BB14 (Colangelo et al., 2008). Previous studies have largely shown tha BB14 is active in vitro and in vivo showing differentiation of dorsal root ganglia, activation of molecular mechanisms dependent on phosphorylation of TrkA, decreased reactive gliosis and reduction of neuropathic pain in animal models (Colangelo et al., 2008; Cirillo et al., 2012). However, the anti-gliosis activity of BB14 needs to be confirmed in vitro. Another objective of this thesis project was to study the role of the purinergic system in neuroinflammation. In fact, it is known in the literature that activated glial cells perturb neuroglial network and synaptic homeostasis through the production and release of

glutamate, ATP, and cytokines, thus contributing to alteration of the glutamate/GABA balance and related uptake mechanisms. It is now generally accepted that high levels of extracellular nucleotides such as ATP may be released under pathological conditions such as inflammation, trauma, and stress. Interestingly, a number of neurodegenerative conditions exhibit enhanced P2X7 receptor expression in the neuroinflammatory loci where activated microglia are a coexisting feature (Skaper et al.,).

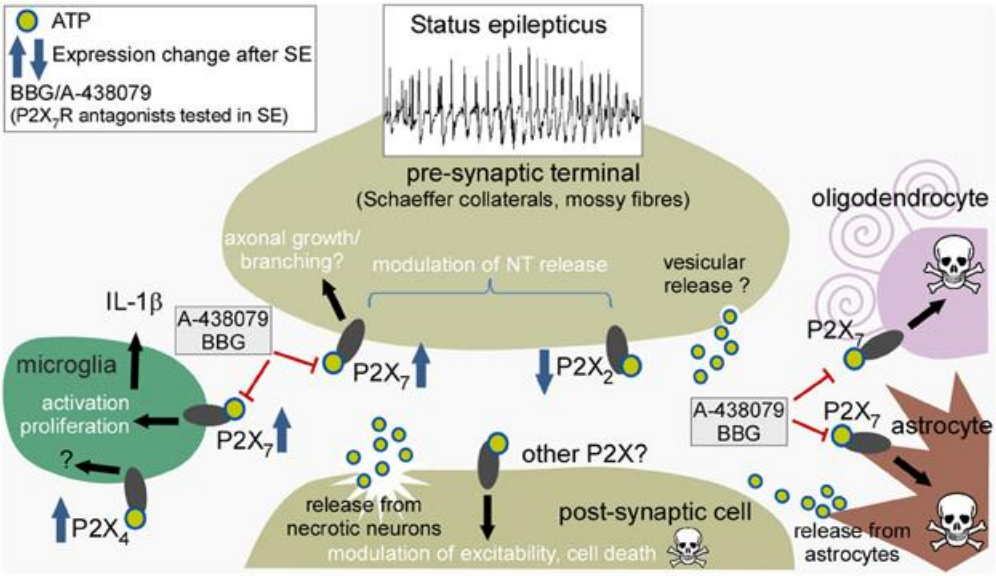


Figure 3.1. Schemathic rappresentation of neuro-glia structure (Henshall et al,. 2013).

We focused on the purinergic P2X7 receptors because they are expressed on both pre- and post-synaptic neurons, as well as on microglia, astrocytes and oligodendrocytes. Several in vivo and in vitro evidence shows that the activation of P2X7R induces the secretion of pro-inflammatory cytokines and chemokines, such as TNFα, and also stimulates the production of superoxide and nitric oxide in microglia cells. These mechanisms are well known in microglia but, less clear is the role of astrocytes. In particular, to study the purinergic system we used OxATP, a non-selective antagonist of P2X7 receptors. Our experiments show that OxATP decreased the astrocytic overgrowth induced by TNFα and LPS stimulation, but did not have effect on neuronal and astrocytic survival, as demonstrated by experiments of BrdU incorporation and MTT assays. OxATP also significantly decreases the astrocytic and neuronal ROS production both in basal conditions and after oxidative stress. WB analysis also showed that treatment with OxATP restores the levels of expression of vGLUT that are drammatically reduced by toxic stimuli. All together these data demonstrate that OxATP, through P2XRs, modulates glial and neuronal glutamate transporters and astrocytic hyperproliferation following toxic and neuroinflammatory stimuli.

Therefore, also P2XRs can be considered a potential target to limit neuroinflammatory process associated with neurodegenerative diseases.

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