Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Biologia Cellulare e Molecolare

Ciclo XXIX

Settore Concorsuale di afferenza: 05/D1

Settore Scientifico disciplinare: BIO/09 FISIOLOGIA

TITOLO TESI

PHYSIOPATHOLOGICAL PROTEIN RELEASE BY GLIAL CELLS: FOCUS ON PURINE NUCLEOSIDE PHOSPHORYLASE (PNP), SUPEROXIDE DISMUTASE 1 (SOD1), AND α-SYNUCLEIN.

Presentata da: Francesca Massenzio

Coordinatore Dottorato   **Relatore**

Chiar.mo Prof. Giovanni Capranico   **Prof.ssa Barbara Monti**

Esame finale anno 2017

INDEX

ABSTRACT

Previous studies from our laboratorory highlighted the neuroprotective role of glial cells against neuronal damage especially through protein release (Polazzi and Monti, 2010). Given the involvement of glial cells not only in the surveillance and homeostasis of the Central Nervous System, but also in pathological conditions, in this thesis we focused our study on the glial release of three different proteins, Purine Nucleoside Phosphorylase (PNP), Superoxide Dismutase 1 (SOD1) and α-synuclein related to different neuropathological conditions, i.e. PNP-deficiency, Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease (PD).

Purine Nucleoside Phosphorylase (PNP) protein is a ubiquitous enzyme, which catalyzes the intracellular cleavage of ribonucleosides to generate purine bases. Physiologically, PNP plays a crucial role in the metabolism of purines and PNP deficiency causes a rare inherited disease determining severe combined immunodeficiency and mental retardation (SCID). PNP histochemical localization is restricted to glial cells, whereas its expression in neurons is still debated. Similarly to human disease, PNP deficiency in mice, due to missense mutations in the gene codifying for this enzyme, showed a reduction in number of immature and peripheral T-cells and in T-cell proliferation. Neurological symptoms have been described in 50% of patients; neurological involvement seems to be related with the severity of enzyme deficiency because GTP is necessary for neurotransmission. Notwithstanding the relevant role of glial cells in PNP-deficiency, the role of PNP in glial cell physiology has never been studied before.

Superoxide Dismutase 1 (SOD1) catalyzes the dismutation mechanism, which is the transformation of superoxide to H_2O_2, which in turn is converted to H_2O by catalase, peroxiredoxin (Prx) or glutathione peroxidase. Catalytic activity is required to maintain low levels of intra and extracellular ROS, superoxide and H_2O_2, indispensable in signaling or in defense against pathogens. If the level of ROS is too high, it may be cause of oxidative stress. A previous published study from our laboratory demonstrated constitutive SOD1 production and release by microglia and the consequent accumulation in

conditioned medium (MCM) with a neuroprotective role of microglia in case of neuronal damage; SOD1 was found to be one of the neuroprotective released molecules. On the other hand, mutations in SOD1 are a key player in the onset of Amyotrophic Lateral Sclerosis (ALS); approximately 20% of familial cases (FALS) are due to mutations in the gene coding for SOD1. Neurodegenerative diseases, stroke, trauma and hypoxia affect neuronal survival indirectly by triggering neuroinflammation. Microglia are then activated (or become reactive) in response to insults acquiring a phagocytic phenotype and releasing inflammatory mediators, cytokines and chemokines. The acute neuroinflammatory response seems to be beneficial to the CNS, since it is an attempt to reduce injury and restore damaged tissues. Because of the dual role of SOD1 and microglial cells, we decided to study the effect of wild-type and mutant SOD1 (G93A and A4V) in glial cell pathophysiology.

Alpha-synuclein is a ubiquitous protein belonging to a family that also includes β- and γ –synucleins. In the brain, α-synuclein represents 0.5-1.0% of all cytosolic proteins and is primarily localized at presynaptic terminals. The protective or toxic effect of α-synuclein depends on its expression levels in the cell: α-synuclein in physiological conditions would have a protective function, while its accumulation and its aggregation into oligomers or fibrils would have a detrimental effect on cells. Some point mutations (A53T, A30P and E46K) in the α-synuclein gene are related to the onset of an autosomal dominant juvenile form of Parkinson's disease. Taking these evidences together and because most studies on α-synuclein were performed on neurons, we decided to analyze as a first step whether α-synuclein could be secreted by glial cells and then we tried to define the conditions of this secretion and its role.

Therefore, the first aim of this thesis was to analyze the release of PNP, SOD1 and α-synuclein by glial cells in physiopathological conditions in *in vitro* models: primary cultures of rat astrocytes and microglia for PNP and SOD1, as well as immortalized mouse astrocytes and microglia cell lines for α-synuclein (respectively IMA2.1 and N9). In particular, for all three proteins the effect of different glial activating stimuli (LPS, IFNγ and ATP) on their release, as well as their vesicular secretion through exosomes were analysed. Considering the role of mutations in the genes codifying for SOD1 and α-

synuclein in familial forms of neurodegenerative diseases, respectively ALS and PD, a second aim was to investigate whether and how overexpression of wild-type SOD1 or mutations linked to ALS (G93A and A4V) could alter the functioning of microglia and astrocytes, determining activation and thereby contribute to inflammation, and whether this condition could affect neuronal survival and/or death. In parallel, a preliminary study on α-synuclein release was carried on by a preliminary investigation on overexpression of wild-type or PD-linked mutant α-synuclein (A53T and A30P) in glial cells.

By using the above-mentioned in vitro approaches, we observed that all brain cells, but mainly microglia and astrocytes, constitutively release PNP. Glial stimulation with potent inflammatory agents, like lipopolysaccharide and interferon-γ, did not modify PNP release, while ATP and Bz-ATP led to a dose-dependent increase of release mediated by $P2X_7$ receptors and occurred through the secretion of lysosomal vesicles in the extracellular medium. The mechanisms activated by ATP seem to be common in pathological conditions such as ischemia, traumatic injury and seizure activity and might suggest a pathological role of extracellular PNP in the mechanisms of PNP-deficiency.

Regarding SOD1, this enzyme is constitutively released by microglial cells and astrocytes, even through vesicles and mutant SOD1 (fALS murations) appears to be less released than wild-type SOD1. Intracellular accumulation of mutant SOD1 in both microglia and astrocytes correlates to dysfunction of autophagy mechanisms and promotes the activation of microglial cells. In addition, we developed a primary co-culture *in vitro* model in order to evaluate the effect of glial cell SOD1 overexpression on neuronal survival and death. We show that microglia exert neuroprotection in cerebellar granule cells exposed to the neurotoxic stimulus glutamate, but microglial mutant SOD1 overexpression appears to be toxic to neurons. Astrocytes, on the other hand, seem to be less susceptible to mutant SOD1overexpression and do not display a neuroprotective function against glutamate excitotoxicity, even if overexpression of wild-type SOD1 seems to provide neuroprotection. Trehalose, a disaccharide known to be able to restore the physiological autophagic flux, showed significant ability in restoring the physiological pathway of SOD1 expression in glial cells and significantly reduced the death

of cerebellar granule neurons due to glutamate excitotoxicity, giving promising expectations for future studies.

Moreover, α-synuclein is constitutively released by microglial cells and astrocytes, even through vesicles. The increased release after LPS but not ATP treatment underlies the different response of glial cells to different stimuli. Since activation of glial cells is strictly related to the progression of neurodegenerative diseases, inflammatory pathways might be responsible for the impairment of glial cell function. According to this hypothesis, we observed that overexpression of α-synuclein mutations linked to Parkinson's disease promoted an increased release of α-synuclein compared to wild-type α-synuclein. Thus, α-synuclein overexpression, even wild-type α-synuclein, promoted the activation of microglia, though not of astrocytes. We could speculate that the increased release, together with increased activation, might be responsible for reduced neuroprotection and for diffusion of potential toxic mutant α-synuclein.

1. INTRODUCTION

1.1 THE CENTRAL NERVOUS SYSTEM

The central nervous system is composed by two different cells type with a ratio of 1 to 10-50; neurons and glial cells or neuroglia (Rezaie et al., 2002). While neurons are specialized in the conduction of electric signals, glial cells do not participate directly in signal transmission, though they play an important role during neuronal development and are also involved in the trophic and protective support of neurons throughout life. In the Central Nervous System (CNS) there are four types of glial cells: ependymal cells, oligodendrocytes, astrocytes and microglial cells.

Ependymal cells, whose functions are not clear, are columnar cells located close to the brain ventricles and the central canal of the spinal cord.

Oligodendrocytes are responsible for the formation of the myelin sheet in the CNS. The myelin sheet acts as an insulator of axonal segments and is indispensable for nerve conduction.

Astrocytes are the most numerous cells in the CNS; they have a high number of ramifications that allow them to contact blood vessels and create the blood-brain barrier.

Microglia are the resident macrophages in the central nervous system. They are involved in the surveillance of the environment; in case of injury or neuronal damage, glial cells are the most responsive cells helping neurons restore physiological conditions (Hartline 2011). Microglial cells represent 5-20% of the glial population in the central nervous system.

Cells of the Central Nervous System
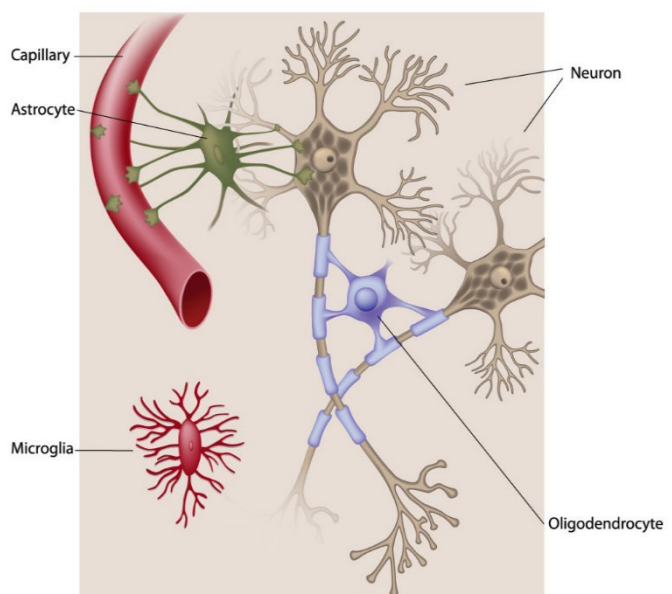
The major brain areas rich in microglia are the hippocampus, telencephalic region and basal ganglia; the cerebellum and brain stem are much less populated (Lawson et al., 1990).

Since glial cells establish numerous interactions with neurons regulating their function, homeostasis and protection from stress and damage, the involvement of glial cells in the most known neurodegenerative diseases is well-established, though it remains to be characterized.

1.1.1 MICROGLIAL CELLS
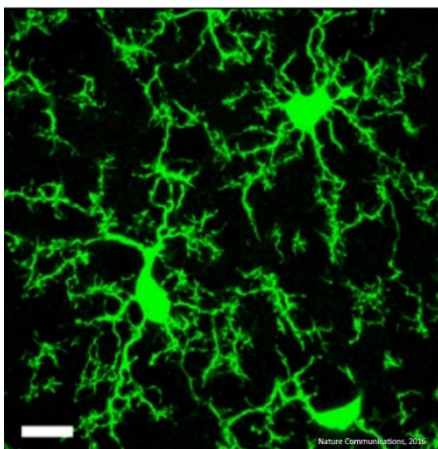
Microglia are the resident mononuclear phagocytes of the CNS (Lawson et al., 1990). Defining the origin of microglia has not been easy and there have been several schools of thought.

The concept of neuroglia was introduced in 1856 by Rudolf Virchow; (Verkhratsk, 2006); he defined "nervenkitt" cells with the ability to form a kind of nervous adhesive tissue, able to keep connected adjacent cells. At the beginning of the nineteenth century, Robertson defined "mesoglia", phagocytic cells with mesodermal origin present in the CNS. Mesoglia mainly correspond to oligodendrocytes, renamed by Santiago Ramon y Cajal "third element of the nervous system" to further differentiate them from neurons (first element) and neuroglia (second element) (Florent et al., 2012). The concept of the third element was clarified by Del Rio-Hortega; he called "microglial cells" the non-neuronal, non-astrocytic third element as distinct from neuroectodermal oligodendroglia or oligodendrocytes (Rezaie et al., 2002).

1.1.1.1 Origin of microglia

Regarding the origin of microglia, there are two well-known hypotheses: the neuroectodermal hypothesis and the myeloid-monocytic hypothesis.

1) The neuroectodermal hypothesis has been supported by studies on the identification of microglial markers during the development of the brain, germinal matrix, central nervous system or in the neuroectoderm.

2) The hypothesis that microglia derives from blood monocytes, originating in the bone marrow and circulating in the CNS between birth and the postnatal period, is the most-approved hypothesis. According to this myeloid-monocytic hypothesis, resident microglia and in general resident brain macrophages, are derived from circulating blood monocytes during embryonic life and in the postnatal period (Polazzi et al., 2010; reviewed by Kettenmann H. et al., 2011). However, data related to microglia during brain development, before vascularization and the formation of macrophages in the neural epithelium from precursors different from the ones originating monocytes, together with the phenotypic differences between mononuclear phagocytes in the embryonic-fetal stage and neonatal-adult period (Takahashi, 2001), suggest that prenatal microglia might arise from mesodermal progenitors.

There is evidence supporting the existence of two different microglial populations during fetal and embryonic development; a population with myeloid/mesenchymal origin, not necessarily monocytes; and a second population, which represents a transient form of fetal macrophages derived from blood precursors, probably monocytes, that are related to amoeboid microglia observed in the post-natal period in rodents (Rezaie et al., 2005b). This second microglial population lays close to blood vessels and can be replaced by precursors that originate in the bone marrow and express the histocompatibility complex II, indicating their monocytic origin (Eglitis et al., 1997; Hickey et al., 1998). The phagocytic ability of the two cell populations is different, in fact a reduced phagocytic activity is observed in unstimulated cells

with branched phenotype, which however is not observed in the amoeboid phenotype (Szabo et al., 2013).

1.1.1.2 Microglial phenotypes

Microglial cells are known for their plastic ability and functional characteristics depending on their activation state; indeed, it is not difficult to observe morphological microglial changes depending on their function (Szabo et al., 2013). In physiological conditions, microglial cells are in a state of non-activation, also call "resting" or "quiescent" state, in which microglial morphology is characterized by numerous processes that originate from the soma with distal arborization (Streit et al., 1999). In conditions of stress, inflammation, injury or particular signals, microglia change their morphology, shifting to an "activated" state (Stence et al., 2001). Cells undergo an expansion of the cell body, ramifications become shorter, less arborized and located around the soma. This morphological change can induce phagocytic functions in microglial cells and an amoeboid ability that allows cells to move towards lesions (Thored et al., 2005).

1.1.1.3 Physiological functions of microglia

It is well-known that microglia, even when in a "resting" state, monitor the brain microenvironment and synapses. Microglial cells, in fact, are essential for the proper functioning of the CNS, since they are a sensor of pathological conditions (Polazzi et al., 2010). Their highly-branched morphology and plasticity ability, allow microglia to supervise the extracellular parenchyma of the CNS and rapidly activate in response to pathological conditions, exerting typical macrophage functions such as phagocytosis (Neumann et al., 2006, Napoli et al., 2009), secretion of pro-inflammatory cytokines (Banati et al., 1993, Gehrmann et al., 1995, Minghetti et al., 1998) and the ablty to behave as antigen presenting cells (Beauvillain et al., 2008, Aloisi, 2011).

The neuroprotective role of microglia is carried out not only as a response to neuropathological threats, but also under physiological conditions. During brain development, microglia support synaptogenesis through the local synthesis of neurotrophic factors, regulating also synaptic transmission and remodeling; the interactions between microglia and neurons contribute to the development of synapses and neuronal homeostasis, also as a result of injury, which arrange for the recovery of neuronal circuits (Polazzi et al., 2013).

During development, microglia have a role in synaptic maturation, while in the adult brain they have a fundamental role in synaptic plasticity. Microglial cells are able to exert neurotoxic and neurotrophic effects because of their activation state. One of the most peculiar features of microglia is the ability to modify their behavior in response to different signals originating from other cells located in the brain parenchyma. This function suggests the ability to promote neuronal cell survival or death depending on the nature of the neural signals that regulate the activation of microglia. Damaged neurons may release or express molecules able to modulate the activation of microglia. This suggests that different types of neuronal death, apoptosis or necrosis, can induce different microglial responses, which can in turn be responsible for different neuronal fate (Polazzi et al., 2001).

Apoptosis or programmed cell death is crucial in CNS development, since it allows the removal of neuronal subpopulations. Apoptotic death is a common mechanism of neuronal removal during development of the nervous system, which occurs only in pathological conditions in the adult brain, such as in neurodegenerative diseases. Microglia are involved in this process in response to various signals derived from neurons triggering the process of programmed cell death through the release of neurotoxic molecules (De la Rosa et al., 2000).

1.1.1.4 Pathological functions of microglia

Loss of physiological functions is associated with the onset of psychiatric disorders or neurodegenerative diseases. Theoretically, every neurological disorder involves inflammation with the consequent activation of

resident microglia, accompanied by an increase in cell number and in phenotypic change, a phenomenon known as "gliosis".

Neurodegenerative diseases, stroke, trauma and hypoxia, affect neuronal survival indirectly by triggering the state of neuroinflammation. Microglia are then activated (or become reactive) in response to insults adopting a phagocytic phenotype and releasing inflammatory mediators, cytokines and chemokines. The acute neuroinflammatory response seems to be beneficial to the CNS, since it is an attempt to reduce injury and restore damaged tissues (Wake et al., 2013, Kiefer et al., 1995, Streit et al., 1999, Imai et al., 1997, Lalancette et al., 2007, Yanagisawa et al., 2008, Madinier et al., 2009).
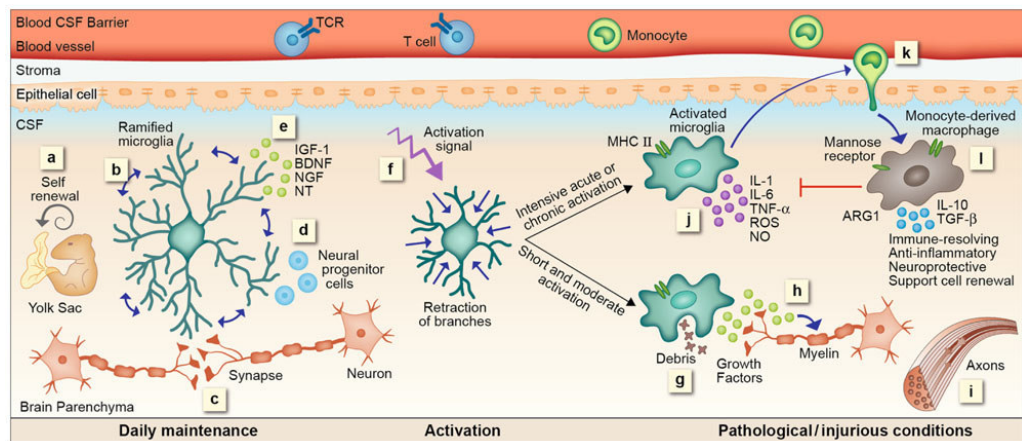
Microglia and monocyte-derived macrophage functions –cascade of events. London et al., Front. Cell. Neurosci., 2013.

On the other hand, chronic neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), tauopathies and multiple sclerosis (MS), are known to be associated with chronic neuroinflammation. Although several differences have been found between these chronic diseases, neuroinflammation is a response to long-term injury or initial insult.

A typical feature of neuropathological conditions is the activation of microglia and the subsequent release of inflammatory mediators leading to an increased oxidative and nitrosative stress. This condition promotes the inflammatory cycle; the duration of the inflammatory response and the activation of microglia in this case are harmful to the nervous tissue (Frank-Cannon et al., 2009, Polazzi et al., 2010).

Activated microglia have two different phenotypes M1 and M2.

1) Classically activated microglia, M1 (LPS or IFNγ) have pro-inflammatory, neurotoxic properties, inhibiting the proliferation of lymphocytes; M1 activated macrophages secrete pro-inflammatory cytokines such as interleukin IL-1α, IL-1β, tumor necrosis factors (TNF) and nitric oxide (NO).

2) Alternatively activated microglia, M2 (IL-4, IL-13), are able to repair small damage and show an anti-inflammatory phenotype, contributing to the trophic support of neurons and have the ability to degrade aggregates and increase neuroprotective functions due to the production of anti-inflammatory interleukins (Geissmann et al., 2008, Hu et al., 2014).

In vitro studies in cell cultures have shown the ambivalent role of microglial cells on neurons; neuroprotective but also neurotoxic, while in vivo studies mainly support the neuroprotective potential of activated microglia (Streit et al., 2008). It has not been clearly defined whether the activation state of microglia is beneficial or detrimental to neuronal survival, though the most accredited hypothesis today is that microglia may have a dual role depending on the nature, duration and extent of injury (Ransohoff et al., 2009).

In case of acute injury, microglia are able to remove damaged neurons through its phagocytic activity. This ability is attenuated by pro-inflammatory cytokines, suggesting that microglia involved in the inflammatory response could have reduced phagocytic activity (Koenigsknecht-Talboo et al., 2005). In neurodegenerative diseases, microglial activation occurs before neuroinflammation. A possible explanation may be that microglial activation induces neuroprotection through the elimination of toxic molecules (Polazzi et al., 2010). Furthermore, because neurodegenerative diseases cause alterations in many other cell besides neurons, not only are neuronal functions altered, but also the cross-talk between different cell types (Appel et al., 2010). In neurodegenerative diseases, the role of microglia in the early stages, seems to be linked to an increase of inflammatory functions resulting in neurodegeneration (Polazzi et al., 2010). However this does not exclude the hypothesis of microglial dysfunction; the loss of neurotrophic/neuroprotective microglial functions induces neurodegeneration (Streit 2014). During aging

and the onset of neurodegenerative diseases, there is a reduction in the phagocytic ability of microglia and in the trophic support to neurons. Therefore, if on one side the primary activation of microglia reflects a physiological mechanism focused on neuroprotection mediated by the secretion of cytokines and neurotrophic factors, the removal of damaged cells (Napoli et al., 2009) and phagocytosis of polymorphonuclear neutrophils (Neumann et al., 2006), on the other side the overactivation or the loss of microglial functions increases preexisting neuropathology (Streit et al., 2009). Alteration of microglial phenotype and as consequence the impairment of physiological functions in glial cells, might promote neuronal damage through a "non-cell autonomous" mechanism (Kathrin et al., 2014). According to this disease mechanism, neurodegeneration is strongly influenced by toxicity or mutant protein expression in non-neuronal cells which could be spread to neighbouring neurons.

In the literature, one of the main methods used to activate microglia both *in vitro* and *in vivo* is treatment with the bacterial endotoxin (LPS) (Hetier et al., 1988). In response to LPS microglia release cytokines, chemokines and NO. This effect is mediated by TLR4, belonging to the Toll-like receptor family (Takeda et al., 2003). Microglial activation, however, is reduced by treatment with anti-inflammatory compounds, followed by the reduction of inducible nitric oxide synthase (iNOS) expression. Microglial activation promotes on one hand the decreased production of reactive oxygen species, while on the other hand it increases the neuroprotective functions of microglia (Nathan et al., 2002). Microglia-mediated neuronal homeostasis has been also found altered in serious neuronal damage, which could be mediated by glutamate excitotoxicity and is involved in the majority of neurodegenerative processes. Glutamate acts via N-Methyl-D-Aspartate (NMDA) receptors and in physiological conditions its excess derived from synaptic activity is employed by astrocytes. In pathological conditions, glutamate uptake by astrocytes is compromised inducing an increase of the intracellular calcium concentration and neuronal cell death. LPS-activated microglia express the GLT-1 protein involved in glutamate uptake (Hanisch et al., 2007). LPS-treatment of hippocampal slices induces the stimulation of microglia and a fast and transient

increase in post-synaptic AMPA-mediated currents in the hippocampal neurons of the CA1 region. This effect was not observed in animals lacking microglia, indicating that LPS stimulation requires microglia and confirming its neuroprotective role. Following stimulation, microglia produce adenosine triphosphate (ATP), which acts as transmitter and recruits astrocytes that release glutamate allowing an increase of excitatory synaptic transmission through the glutamate metabotropic receptors (Iadecola et al., 2007).

1.1.2 ASTROCYTES

Astrocytes are the most numerous cells of the CNS (Parpura et al., 2012). In the human brain, the number of astrocytes was estimated to be more than five times the number of neurons (Hartline et al., 2006). During CNS development, the generation of astrocytes is subsequent to the initial production of neurons.

Astrocytes exert several important functions during the development of gray and white matter. They are also responsible for the formation and function of synapses during development, thanks to the release of molecular signals, such as thrombospondin. At the end of the nineteenth century, astrocytes were distinguished in protoplasmic and fibrous astrocytes based on morphological and anatomical differences. Protoplasmic astrocytes have been found in the gray matter and wrap around synapses through numerous branched processes, fibrous astrocytes have been found in the white matter and contact the nodes of Ranvier through long thin fiber-like processes. Both types of astrocytes establish direct contact with blood vessels and form gap junctions between the distal processes of
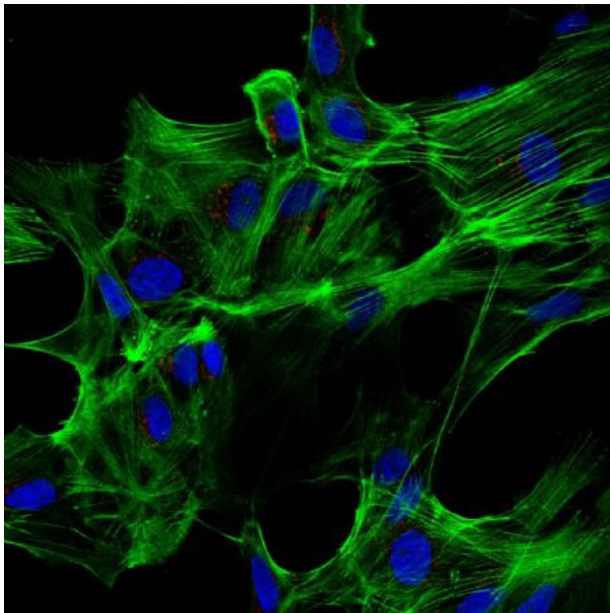
neighbouring astrocytes. At the end of the nineteenth century, Camillo Golgi theorized that astrocytes could be connected to blood vessels (Golgi 1871). However, only recent studies have highlighted the dynamic processes supporting these structural interactions, such as the dialogue between astrocytes and other vascular elements. Astrocytes have an ordered arrangement and a minimal overlapping, and each one interfaces with blood vessels and includes hundreds of synapses (Bushong et al., 2004). The cell surface is covered by lamellar extensions and protrusions which make astrocytes able to interact with surrounding synapses. Most astrocytes possesses the so called "end-feet", contact blood vessels and contribute to the formation of the blood-brain barrier (BBB), a diffusion barrier that prevents the influx of foreign molecules into the brain parenchyma (Simard et al., 2001).

1.1.2.1 Physiological role of astrocytes

Astrocytes play essential functions in the CNS in physiological conditions thanks to the processes able to contact synapses, axons at the nodes of Ranvier, neuronal cell bodies and blood vessels at the endothelial cell layer. The connections with blood vessels allow astrocytes to collect glucose for energy supply, adjust blood flow and the cerebral homeostasis of ions and transmitters (Iadecola et al., 2007). Astrocytes play a synaptic role during development, but also in the adult brain, especially in remodeling and synaptic pruning: they are involved at GABAergic and glutamatergic synapses in neurotrasmitter uptake to prevent excitotoxicity by glutamate and play a direct role in synaptic transmission by releasing molecules synaptically active such as purines (ATP and adenosine), γ-aminobutyric acid (GABA) and D-serine, modulating cell excitability and increasing the concentration of the intracellular calcium ion (Ca2 +) (Shimizu et al., 2007, Bittner et al., 2010). D-serine binds to post-synaptic NMDA receptors (NMDARs), which also bind glutamate released from the pre-synaptic terminal. These events facilitate NAMDAR activation and induction of synaptic plasticity (Verkhratsky et al., 2010). Astrocytes modulate synaptic function through cross talk and exchanging gliotransmitters, ions, second messengers and nutrients through gap junctions

(Parpura et al., 2009, Ullian et al., 2004). These observations support the concept of "tripartite synapses", according to which astrocytes represent the third unit element of signal transmission together with presynaptic and postsynaptic terminations (Volterra, 2005). The main role of astrocytes in this "model" is to isolate and support the function of individual synapses, ensuring spatial specificity in synaptic transmission (Hewett et al., 2009).

Astrocytes play also a metabolic role, since they are responsible for the storage of glycogen granules in the CNS, especially in high-density synaptic areas. In conditions of hypoglycemia and high neuronal activity, glycogen is able to support cell activity. Glycogen stored in astrocytes may provide lactate, which once transferred to adjacent neural elements can be used as aerobical fuel by neuronal mitochondria (Bélanger et al., 2011). The involvement of astrocyte glycogen stores is required for the formation of long-term memory in rats (Pellerin et al., 2007).
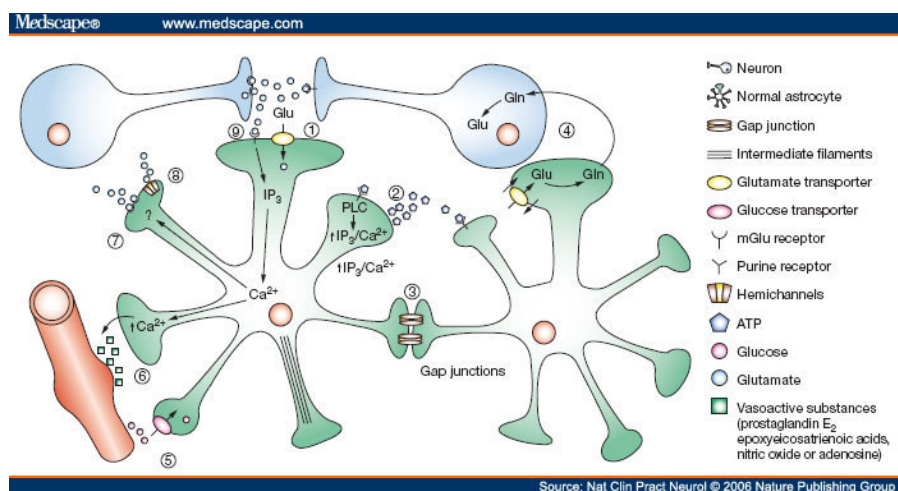
Astrocyte functions. (Maragakis et Rothstein, 2006)

Furthermore, although not presenting action potentials along their processes, astrocytes express sodium and potassium channels which may be involved in evoked currents and show forms of excitability, for example in the case of Ca2+ concentration increases. These increases are necessary for the intracellular communication that is established between astrocytes and between astrocytes and neurons (Molofsky et al., 2012).

Astrocytes have role in every aspect of neuronal function, through the regulation of blood flow, supply of energy substrates and modulation of synaptic function and plasticity, therefore damaged astrocytes are the primary cause of serious neural dysfunction (Maragakis et al., 2006). Following specific acute injuries, inflammation, chronic neurodegenerative diseases or stress, astrocytes respond through the reactive astrogliosis process and the expression of glial fibrillary acidic protein (GFAP), a sensitive and reliable marker for the immunohistochemical identification of reactive astrocytes. In the same way as microglial activation, reactive astrogliosis is not an on-off phenomenon, but rather a continuum event modulated by changes in the surrounding microenvironment and is regulated by specific inter and intracellular signaling (Yamanaka et al., 2008). Depending on the severity of the insult, reactive astrogliosis leads to a series of changes in astrocytes at the molecular, cellular, and functional (gain or loss of function) level, affecting adjacent cells in a beneficial or harmful way. Reactive astroglial cells play several essential beneficial functions in response to CNS insults, such as repairing the BBB, neuroprotection and limiting the spread of inflammatory cells. Loss or alteration of these functions in the astrogliosis process produces neuronal dysfunction and could be a primary cause of disease in the CNS such as neuropathology, psychiatric disorders, trauma and stroke. Animal models have shown that loss of astrocyte function leads to altered CNS function such as excitotoxicity-induced neurodegeneration, caused by an altered uptake of glutamate or increased inflammation and infection due to the loss of astrocyte barrier functions. Following specific inflammation signals, such as LPS exposure, a gain of harmful functions may also occur in reactive astrocytes, such as the inhibition of axonal regeneration, production of reactive oxygen species (ROS), glutamate release and the impairment of the BBB functionality. Moreover, the involvement of other cell types, such as microglia, macrophages and lymphocytes, stimulates astrocytes to produce cytotoxic levels of molecules which are normally non-toxic and contribute to inflammation and chronic neuropathic pain (Verkhratsky et al., 2010, Molofsky et al., 2010,

Rappold et al., 2010). Understanding these processes could simplify treatment strategies to preserve the beneficial role of astrocytes. Recent studies have shown that astrocytes are the main players in many developmental diseases, such as autism and schizophrenia, characterised by defects in synaptic formation (Parpura et al., 2012, Sofroniew et al., 2009). In fact, in the development of white matter, dysfunction or malfunction of astrocyte connections or gap junctions leads to demyelination. Given their strong involvement in developmental diseases, astroglial cells may be a useful target in the development of new therapies. The functionality of astrocytes is altered not only in neurodegenerative diseases, such as ALS, Alzheimer's and Parkinson's disease, but also in neurodevelopmental diseases such as Rett syndrome, X-fragile, Alexander's disease, epilepsy, autism, psychiatric disorders and in brain tumors.

1.1.3 NEURON-GLIA INTERACTIONS

Glial cells represent about 90% of all cells in the human brain. The initial role as brain "glue" has been replaced with a more important role of key players in brain physiology, metabolism, development, and even neurological diseases (Barres, 2008). An essential aspect of normal brain function is the bidirectional interaction and communication between neurons and neighbouring glial cells; dysregulation of this interaction may contribute to the onset of neurodegenerative diseases. Together with astrocytes, microglia can release neuromodulatory molecules involved in intracellular signalling and the communication with neurons.
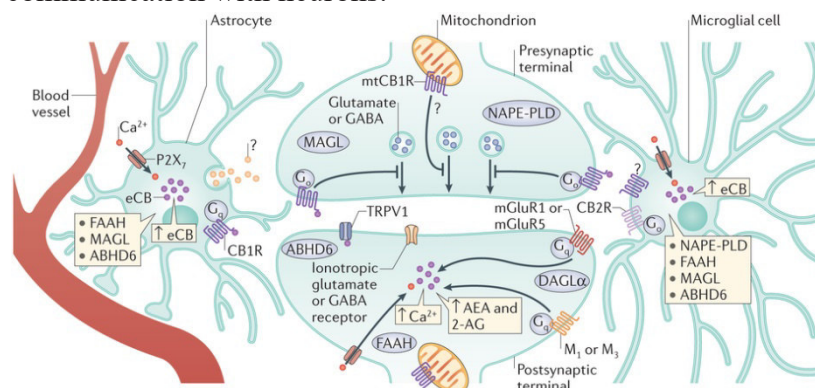
Nature Reviews Neuroscience 16, 705–718 (2015).

The first evidence of interaction between microglia and neurons is given by the complementary expression of the microglial receptor for chemokines (CX3CR1) and its neuronal ligand (CX3CL1), thus suggesting the ability of microglia to modulate neurotransmission (Béchade et al., 2013). The CX3CL1/R1 pathway seems to be involved in synaptic maturation and an alteration of this pathway can lead to delayed glutamatergic synaptic maturation and alteration of hippocampal development (Paolicelli et al., 2011). Another example of neuron-microglia interaction is related to the CD200R membrane protein. CD200R is a membrane receptor exclusively expressed by microglia while its ligand, CD200 is expressed by neurons, oligodendrocytes and astrocytes. CD200-deficient mice display LTP inhibition, further underlining the role of microglia in neuronal homeostasis and synaptic plasticity (Costello et al., 2011). In fact, microglia is a sensor of neuronal activity modulating most contacts between neurons and synaptic elements (Tremblay et al., 2011, Wake et al., 2013). The signaling modulating this function is not known, though it seems to be mediated by the secretion of signaling molecules by microglia, including cytokines (Elkabes et al., 1996, Hanisch, 2002) and neurotransmitters (Hanisch, 2002, Flierl et al., 2007), which regulate synaptic functions. *In vivo* studies suggest a role of microglia in the regulation of hippocampal (Battista et al., 2006, Ziv et al., 2006) and sub ventricular area neurogenesis (Thored et al., 2009) after stroke. In an *in vitro* co-culture model of neural stem cells with microglia or conditioned medium from microglia, the secretion of factors by microglia needed for neurogenesis was oberved (Walton et al., 2006). Microglia-neuron interaction and the consequent regulation of neuronal homeostasis by microglia were also found in severe neuronal damage, such as glutamate excitotoxicity. Glutamate acts via N-methyl-D-aspartate (NMDA) receptors inducing an increase of neuronal intracellular calcium concentration, and consequent neuronal death. Neurotoxicity due to glutamate is involved in numerous neurodegenerative processes, such as ischemia and brain injury. In physiological conditions, the excess of glutamate resulting from synaptic activity is uptaken by astrocytes, while in pathological conditions, glutamate uptake by astrocytes is compromised. LPS-activated microglia express the GLT-1 protein implicated

in glutamate uptake (Hanisch and Kettenmann, 2007). Microglia stimulation with lipopolysaccharide (LPS) treatment of hippocampal slices, induces a rapid and transient increase in the spontaneous post-synaptic AMPAergic current in hippocampal CA1 neurons. This effect has not been found in animal models with microglia depletion, indicating that LPS stimulation requires microglia and confirming the neuroprotective role of microglial cells. Following this stimulation, microglia recruit astrocytes through the production of ATP. Astrocytes release glutamate allowing an increase of excitatory synaptic transmission through metabotropic glutamate receptors (Pascual et al., 2012).

Astrocytes express neurotransmitter receptors, but at the same time, thanks to the increase in calcium concentration, they release neurotransmitters (ATP, Glutamate, D-serine, and GABA) essential for the modulation of neuronal activity (Haydon, 2001, Volterra, 2005) Glutamate release from astrocytes seems to be a key modulator of synaptic transmission depending on the kinetic of calcium elevations. Slow elevation in astrocytes induces slow inward currents in CA1 neurons by acting on post synaptic NMDA receptors (Fellin et al., 2004, Angulo et al., 2004). Faster calcium elevation induces presynaptic modulation of synaptic release, by acting on presynaptic mGluR5 receptors (Shigetomi et al., 2008). Activation of glutamatergic receptors on astrocytes, induces the release of D-serine, which acts as a co-agonist of NMDA receptors (Mothet et al., 2005). D-serine is synthetized by astrocytes from L-serine by serine racemase (SR), mainly expressed in glial cells and overexpressed in activated glial cells increasing the production and release of D-serine (Wu et al., 2004). Released D-serine promotes the production of reactive oxygen species (ROS) and the overactivation of NMDA receptors exacerbating glutamate effects and neuronal damage (Mustafa et al., 2010).

1.1.4 GLIAL CELL SECRETION

1.1.4.1 Glial vescicular release mediated by physiopathologycal stimuli

The correlation between neuroinflammation and neurodegeneration has not been yet clarified. Many physiological CNS functions involving glia,

including neurodevelopment, memory maintenance, neuroprotection, homeostatic and metabolic mechanisms, may be mediated by direct cell-cell contact or by the secretion of molecules in a paracrine way both through the release of soluble factors and through extracellular vesicles (EVs), able to transfer potential toxic, aberrant, non-folded or neuroprotective molecules to neighbouring cells.

In 1909, Hans Held observed granular inclusions in glial processes (Held, 1909). A year later, Jean Nageotte observed secretory granules in glial cells of the grey matter indicating an active secretion from these cells (Nageotte, 1910). Alois Alzheimer called these granules, *gliosomes*. Glial cell secretion includes neuotransmitters, neuromodulators, hormones, peptides, metabolic substrates, ROS, growth factors, plastic factors, inflammatory factors and neuroprotective molecules. The release of these molecules is mediated by several pathways; vesicle-based exocytosis of D-serine (Martineau et al., 2013) or glutamate (Montana et al., 2009), diffusion through plasmalemmal pores/channels, release of ATP and/or glutamate through anion channels, $P2X_7$ receptors (Cotrina et a., 1998, Suadicani et al., 2006) and extrusion through plasmalemmal transporters (Unichenko et al., 2013).

Microglia and astrocytes exert their immune function mainly in the inflammatory process following acute and chronic neurodegenerative diseases, among which psychiatric disorders are included (Shie et al., 2011). In these pathological conditions, glial cells become activated (Skaper et al., 2012; Collins et al., 2012; Eikelenboom et al., 2012) and exert their function by releasing several factors.

ATP represents one of the most important neuro/glio-transmitters; during pathology, ATP is released from damaged cells and acts both as a cytotoxic factor and a proinflammatory mediator, being a universal "danger" signal (Franke et al., 2012).

D-serine upregulates glial activation (Panatier et al., 2006). Glutamate excitotoxicity is one of the main events responsible of neuronal degeneration and motor neuron death in Amyotrophic Lateral Sclerosis (ALS) and the impairment in astrocyte uptake of glutamate could be responsible for this event (Ilieva et al., 2009). Release from neural cells could be altered by glial cell

activation through neuroinflammatory stimuli; bacterial LPS and interferon- γ (IFN-γ), which are able to induce a wide range of inflammatory activities, increase phagocytosis, chemotaxis, cytokine secretion, activation of the respiratory burst and induction of nitric oxide synthase (Zielasek, Hartung, 1996), while the neuro/glio-transmitters ATP, which is released by injured and dying neural cells, leads to activation of $P2X_7$ receptors in glial cells (Franke et al., 2006; Weisman et al., 2012; Skaper et al., 2010).

Polazzi et al. showed that neuroprotection is due partly to molecules with a molecular weight lower than 30kDa, such as TGF-β2, SOD1 and ApoE. Transforming growth factor-beta 2 (TGF-β2), as well as SOD1 are constitutively released by microglia and neuroprotection could derive partly from the interaction between microglial released TGFβ-2 or SOD1 with neuronal receptors (Polazzi, 2013).

1.1.4.2 Vescicule-mediated release of proteins

It is known that glial cells signal to neighbouring cells by releasing molecules via regulated exocytosis through lysosomes (Verderio et al., 2012; Bräuer et al., 2004) and glial cell activation, commonly occurring in most neurodegenerative diseases and affecting protein release from these cells by influencing their secretory pathways (Bianco et al., 2005; Hanamsagar et al., 2011). Glia-cell release of molecules may be mediated by extracellular vesicles that can be classified according to size, cargo and origin.

Extracellular vesicles (EVs) comprise shedding microvesicles (MVs), exosomes, and apoptotic bodies. Apoptotic bodies are released during apoptosis, whereas the other types of vesicles are derived from healthy cells (Cocucci et al., 2009, Thery, 2011). Exosomes contain proteins arising from the plasma membrane, endocytic pathway and cytosol. They contain limited amounts of proteins from other intracellular compartments (nucleus, endoplasmic reticulum, Golgi apparatus). RNAs are transported by EVs from cell to cell and can modulate gene expression in the recipient cell. In the immune system, MHC-peptide complexes are secreted by exosomes from

antigen presenting cells (APCs) suggesting a role for exosomes in the adaptive immune response.

Communication within the nervous system could be mediated by EVs; exosomes are secreted by neurons, Schwann cells, astrocytes and microglia (Potolicchio et al., 2005) and they may be responsible of the transfer of pathogenic proteins such as prions, beta-amyloid peptides, superoxide dismutase 1, tau and α-synuclein between glial cells and neurons promoting neuronal damage.
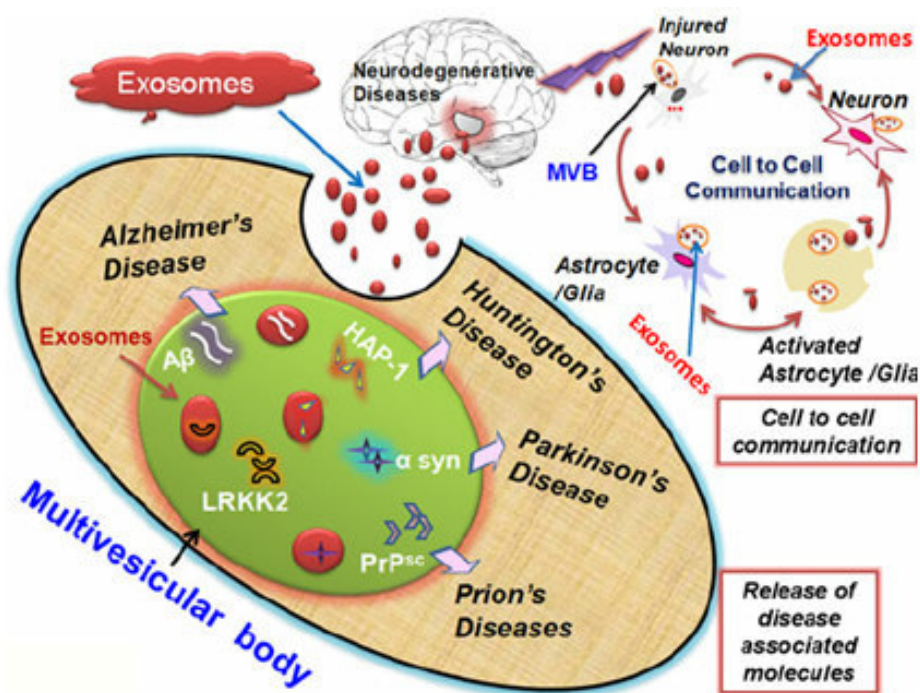
Exosomes: Mediators of Neurodegeneration, Neuroprotection and Therapeutics (2013).

Activation of microglia, mediated by ATP (activation of P2X7 receptors), seems to promote the release of microvesicles with irregular shape and size (0,1-1µm), carrying pro-inflammatory cytokines to recipient neurons increasing inflammation (Verderio et al., 2012). Similar to microglia, astrocyte-derived MVs are increased by activation of P2X7 receptors though these vesicles can be up to 8 µm in size and carry intact mitochondria and lipid droplets (Falchi, 2013).

While microglia appear to be mainly involved in the spread of inflammatory molecules, astrocytes seem to be mainly implicated in the propagation of pathogenic proteins in neurodegenerative disorders. Astrocytes expressing mutant SOD1 (copper-zinc superoxide dismutase 1) secrete

25

increased amounts of exosomes, carrying mutant SOD1. These vesicles can transfer mutant SOD1 to neurons and induce motor neuron death suggesting a role of EVs in the pathogenesis of ALS (Basso et al., 2013). Exposure of astrocytes to amyloid peptide triggers the release of pro-apoptotic exosomes that contain ceramide and PAR4 (prostate apoptosis response 4). These exosomes are taken up by astrocytes and promote their apoptosis suggesting that exosome-mediated astrocyte death may contribute to neurodegeneration in Alzheimer's disease (Wang et al., 2012).

1.2 PURINE NUCLEOSIDE PHOSPHORYLASE (PNP)

Purine Nucleoside Phosphorylase (PNP) is a ubiquitous enzyme, which catalyzes the intracellular cleavage of ribonucleosides to generate purine bases. Physiologically, PNP plays a crucial role in the metabolism of purines (Bzowska et al., 2000) and its deficiency causes a rare inherited disease determining severe combined immunodeficiency (SCID) (Markert, 1991).

PNP protein, a trimer of approximately 90,000 daltons, has been found in most body tissues, showing the highest levels in lymphoid tissue, thus explaining why immune cells are predominantly impaired in PNP deficiency. Accordingly, PNP inhibitors have been developed as T-cell selective immune-suppressants and to treat many forms of leukemia (Balakrishnan et al., 2010; Robak et al., 2006; Bantia, Kilpatrick, 2004). PNP knock-out mice recapitulate most pathological features of PNP-deficiency (Arpaia, et al., 2000). In this animal model, TAT-mediated intracellular delivery of PNP corrects the enzyme deficiency (Toro, Grunebaum, 2006). Concerning neurological defects in brains from PNP-KO mice, morphological abnormalities and apoptosis were detected in the cerebellum, which was smaller than normal with reduced Purkinje cell number (Mansouri et al., 2012).

Despite all data concerning PNP involvement in brain physiopathology, little is known regarding its specific brain cell distribution. PNP histochemical localization is restricted to glial cells, whereas its expression in neurons is still debated (Castellano et al., 1990). Additionally, Dalmau and collaborators

(1998) suggested that microglial cells could be crucial for the development of normal central nervous system (CNS) by controlling purine metabolism. They showed different PNP staining depending on microglial morphology and observed PNP labeling in blood vessels, some nerve fibers and astrocytes, suggesting a complex mechanism of cell interactions in the regulation of extracellular purine levels.
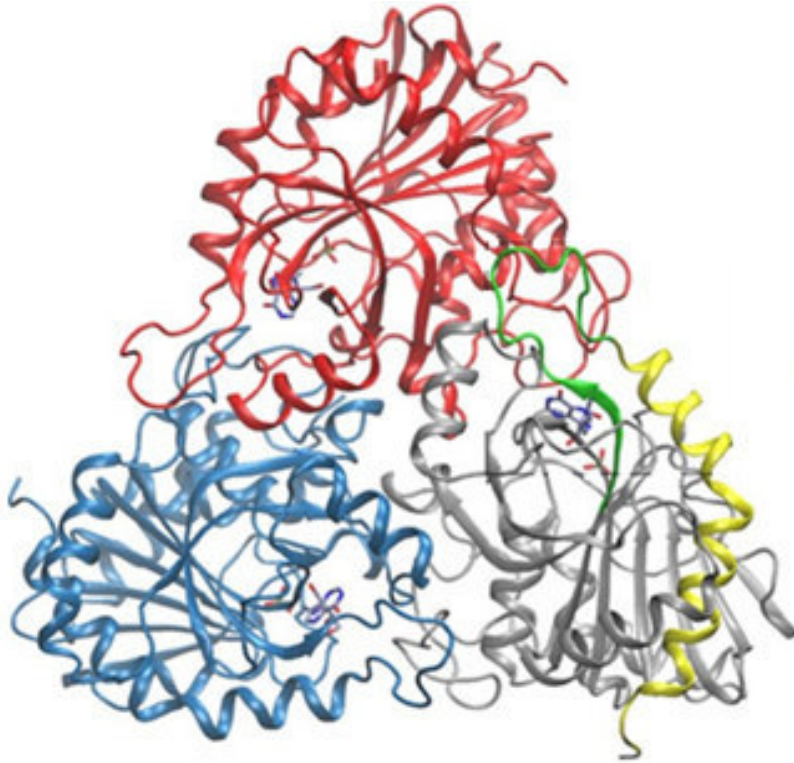
Structures of the human PNP trimer (Nature communication, 2015)

1.2.1 PNP DEFICIENCY

Severe Combined Immunodeficiency (SCID) is a heterogeneous disease characterized by a prenatal disorder in the development of T lymphocytes leading to profound abnormalities of cell-mediated immunity, antibody deficiency, severe viral, bacterial and fungal infections, chronic diarrhoea, failure to thrive and skin rashes as typically seen in Omenn syndrome (OS). This pathology was first described in a five-year old child with T-cell immunodeficiency (Giblett et al., 1975). SCID is caused by mutations in different genes mainly involved in the development and function of both T and

B cells; natural killer cells are present in 50% of patients with SCID and are able to protect against viral, bacterial and fungal infections.

PNP deficiency is a rare form of SCID (Markert, 1991). As in the human disease, PNP deficiency in mice, due to missense mutations in the gene codifying for this enzyme, showed a reduction in numbers of immature and peripheral T cells and in T cell proliferation (Snyder et al., 1997). Furthermore, mutations in PNP caused loss of deoxyguanosine kinase activity. PNP-deficiency could be due to block of purine recovery as a consequence of GTP depletion or to the accumulation of PNP substrates inosine, guanosine, deoxyinosine, and deoxyguanosine.

The abnormal accumulation of dGTP preferentially in mitochondria, inhibits mitochondrial DNA synthesis as a first step of T- lymphocyte apoptosis (Young, 1994).
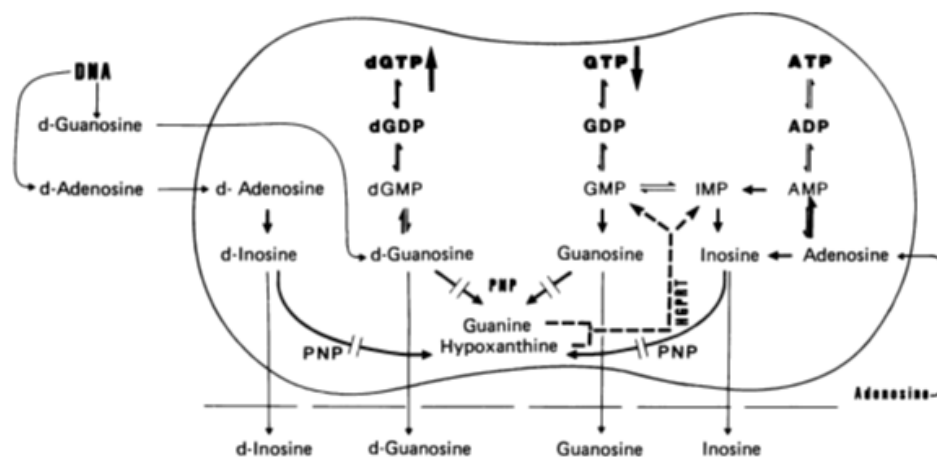
Pathways of purine metabolism, Archives of Disease in Childhood, 1987.

Neurological symptoms have been described in 50% of patients; neurological involvement seems to be related with the severity of PNP deficiency because GTP is necessary for neurotransmission. These evidences support a direct relation between neurological dysfunction and PNP deficiency.

1.3 SUPEROXIDE DISMUTASE 1 (SOD1)

Superoxide dismutase (SOD) is an enzyme that exists in three isoforms: SOD1 (CuZnSOD); SOD2 (MnSOD); SOD3 (extracellular SOD); each one is produced from a different gene with distinct localization, but share the same

function. This distinct localization is important for the compartmentalization of redox reactions.

This enzyme catalyzes the dismutation mechanism, which is the transformation of superoxide by SOD to H2O2, in turn converted in H2O by catalase, peroxiredoxin (Prx) or glutathione peroxidase (GPx) (Go, Jones D, 2011). The conversion involves alternative reduction and oxidation of a transition metal, such as copper (Cu) and manganese (Mn) that are located in the active site of the enzyme (Miao, St Clair, 2009; Zelko et al., 2002; Yamakura, Kawasaki H, 2010; Ushio-Fukai, 2011). Catalytic activity is required to maintain low levels of intra and extracellular ROS, superoxide and H_2O_2, indispensable in signaling or in defense mechanisms against pathogens. If the level of ROS is too high, it may be cause of oxidative stress.

SOD1 is a 153 amino acid homodimer ubiquitously expressed, mainly in the cytoplasm. Each SOD1 subunit binds to a zinc (Zn) and a copper (Cu) atom (Pasinelli, Bown R, 2006; Bruijn et al., 2004). The genomic sequence coding for SOD1 has high similarity among rat, mouse and human, thus indicating a considerable conservation for this gene.
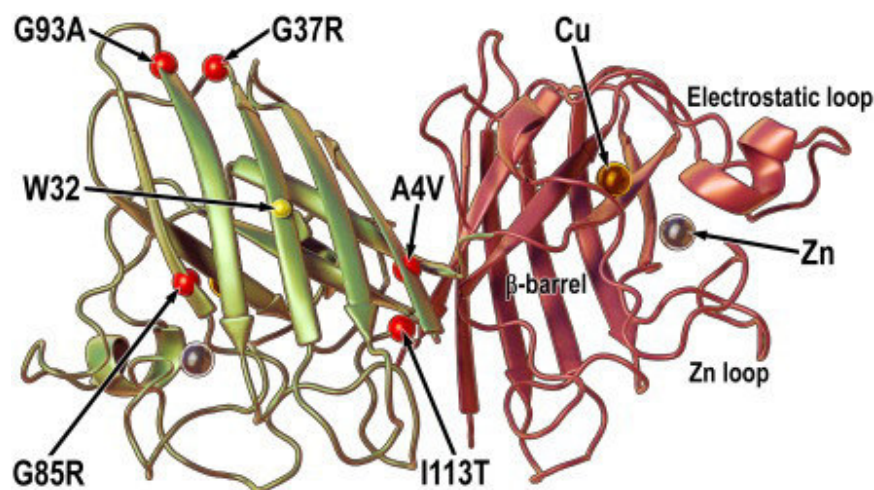
Philbert Ip., 2011

Polazzi et al. demonstrated a constitutive SOD1 production and release by microglia and the consequent accumulation in microglia conditioned medium (MCM). SOD1 was also found among the low molecular weight neuroprotective molecules released by microglia. Treatment of primary cerebellar granule neurons with 6-hydroxydopamine (6-OHDA) induced death of 50% of cells in 24h, however, co-exposure to MCM was able to restore cell

viability. The neuroprotective ability of SOD1 was confirmed by exposing primary cerebellar granule neurons to exogenous SOD1 in presence or absence of SOD1 inhibitors; neuroprotection was abrogated by SOD1 inhibitors. Polazzi et al. also demonstrated an ATP-mediated increase of SOD1 release by microglia, confirming the involvement of the lysosomal secretory pathway (Polazzi et al., 2013).

Mutations in SOD1 are a key player in the onset of Amyotrophic Lateral Sclerosis (ALS); approximately 20% of familial cases (FALS) are due to mutations in the gene coding for SOD1. More than 125 mutations have been identified, 114 of them cause the disease, six are silent and five are intronic variants that do not lead to ALS. Although most of the 114 mutations are missense, twelve mutations are deletions or nonsense mutations that produce truncated proteins; all these mutations are specific to FALS and they have been found only in 1% of sporadic ALS (SALS). The majority of mutations cause reduced dismutation activity while few maintain full catalytic function. There is no clear correlation between enzyme activity and clinical disease progression, therefore it is still unclear how mutant SOD1 leads to motor neuron degeneration (Pasinelli, Bown, 2006). However, it is known that SOD1-mediated toxicity in ALS is not due to a loss of function rather to gain of one or more toxic properties that are independent from SOD1 activity (Lewis et al., 2014).

1.3.1 AMYOTROPHIC LATERAL SCLEROSIS (ALS)

Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease, Charcot's disease (from the French neurologist who first described it in 1860) or Lou Gehrig's disease (Pasinelli and Brown R.H, 2006), is a progressive, lethal, neurodegenerative disease, leading to paralysis of the voluntary muscles. ALS affects adults of both sexes around 55 years; the incidence and prevalence of ALS are respectively 1-2 and 4-6 per 100,000 cases every year (Pasinelli P, and Brown, 2006). Mutations in superoxide dismutase 1 (SOD1), valosin containing protein (VCP), ubiquilin 2

(UBQLN2), charged multivesicular body protein 2b (CHMP2B), optineurin (OPTN), TAR DNA-binding protein 43 (TDP43), fused in sarcoma (FUS), chromosome 9 open reading frame 72 (C9ORF72) have been identified and promote the definition of proteinopathy for ALS (Takeuchi et al., 2015). A hallmark of neurodegenerative proteinopathies is the formation of misfolded protein aggregates that cause cellular toxicity. A key feature of misfolded protein diseases is the ability of the pathogenic protein species to propagate in a prion-like manner to neighbouring cells and to initiate or promote damage.

Approximately 90-95% of ALS cases are sporadic (SALS), the remaining 5-10% are hereditary, familial ALS (FALS), 20% caused by dominant mutations in the gene coding for Cu/Zn superoxide dismutase 1 (SOD1, chromosome 21q22.11). In ALS patients, more than 150 pathogenic mutations in SOD1 have been identified. Despite the involvement of several genes in FALS and SALS, there are similarities between all ALS forms: progressive degeneration of lower motor neurons (atrophy, cramps, twitching) and death of cortical neurons, suggesting a common pathogenic pathway (Grada et al., 2014). Motor neuron degeneration leads to impairment of neuromuscular function, paralysis of skeletal muscles, paralysis of breathing muscles (diaphragm and intercostal muscles) that culminate in respiratory failure, which causes death (Pasinelli and Brown, 2006). The main pathological hallmark of ALS is the atrophy of motor neurons but accumulation of phosphorylated neurofilament inclusions and the deposition of inclusions (spheroids) in axons, known as Lewy bodies, as well as activation and proliferation of astrocytes and microglia are observed (Hall et al., 1998; Wilms et al., 2001). Unfortunately, there is currently no therapy available for this neuropathology; the only approved drug, riluzole, only prolongs the life of patients by a few months (Pasinelli and Brown, 2006; Lewis et al., 2014). Therefore, the characterization of the cellular and molecular processes involved in the onset and progression of the disease is necessary to identify new potential targets useful for therapeutic strategies, as well as for the identification of biomarkers for early diagnosis and therapeutic follow-up.
When the disease affects upper motor neurons, it is defined as primary lateral sclerosis (PLS). The majority of patients affected by PLS develop consequent

problems in lower motor neurons (MND), satisfying the criteria for the diagnosis of ALS. Impairment in lower motor neurons (MND) classified the pathology as progressive muscular atrophy (PMA), though disease progression is not different from ALS. Frontotemporal lobar degeneration (FTLD) affects 3 or 4 persons/100,000 per year and is characterized by abnormal behaviour and language. The main hallmark of FTLD are inclusions containing TAR DNA-binding protein 43 (TDP-43), similar to the ones found in the motor neurons of ALS patients.

In physiological conditions, TDP-43 is located in the nucleus while in the pathology the phosphorylated protein and C-fragment aggregates are located in the cytoplasm. The presence of both TDP-43 and mutant SOD1 aggregates in FALS is controversial; there are groups supporting the absence of TDP-43 aggregation (Al-Chalabi and Leigh, 2005; Chio et al., 2009) and groups supporting the presence of these aggregates (Wills et al., 2009). These evidences may classify ALS and FTLD as a different manifestation of the same disease spectrum.

Nature Reviews | Neuroscience

Interaction between ALS and FTD. Robberecht & Philips. Nature Reviews Neuroscience 14, 248-264.

The mechanism involved in the onset of ALS is not clearly defined; cellular degeneration, protein misfolding, presence of aggregates in the cytoplasm, ROS, excitotoxicity, impairment of axonal transport and abnormal calcium metabolism could be due to the loss of physiological function or gain of toxic function in the proteins responsible of the disease.

Recent studies suggest a correlation between exposure to solvents, pesticides, metals, chemical compounds and the onset of ALS regarding the neurotoxic properties of these substances. (Bonvicini et al., 2010).
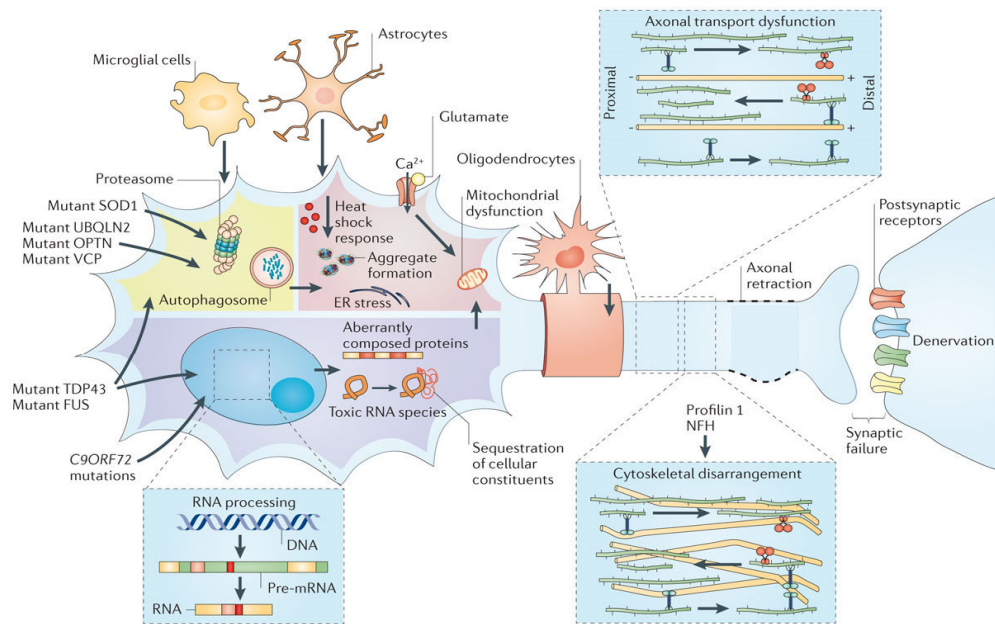
ALS Neurodegeneration mechanisms. Robberecht & Philips, Nature Reviews Neuroscience 14, 248-264.

1.3.2 MUTANT SOD1 IN ALS

As previously described, microglia may show a direct neuroprotective effect against neuronal damage, as well as through the involvement of small neuroprotective molecules released such as SOD1.

The function of SOD1 is controversial; neuroprotective in physiological conditions, but responsible for the onset of ALS, if mutated.

Many hypotheses have been considered about the role of SOD1 mutations in ALS. SOD1 mutations could i) alter the enzymatic activity of SOD1 through the copper aberrant catalysis or the binding of a wrong metal due to an alteration in the configuration of the active channel, or ii) cause a rapid reduction in the bond between SOD1 and copper resulting in the formation of superoxide anion (Bruijn et al., 2004), or iii) give SOD1 an unstable conformation promoting the aggregation of the protein. Protein aggregates increase with disease progression (Johnston et al., 2000; Wang et al., 2002).

The G93A mutation is the most common SOD1 mutation in Europe and is also the most studied one, especially in mouse models. Substitution of G93 is predicted to destabilize the β -barrel (Hart et al., 1998).

Transgenic mice overexpressing human SOD1 G93A mutation show the first symptoms at 3 months of age but the severity of the disease depends on the expression levels of the mutant protein. The progressive weakness characteristic of these mice coincides with microglial activation, astrogliosis, loss of motor neurons in the spinal cord and intracellular aggregates both in motor neurons and glia, thus well-representing the human disease. The inhibition of the proteasome ability to remove aberrant proteins seems a sufficient condition to induce the formation of aggregates in glial cells expressing mutant SOD1 (Bruijn et al., 2004).

These evidences led to a "non-cell autonomous" hypothesis for neurodegeneration in ALS, in which non-neuronal cells of the brain play a central role in inducing neuronal death (Wang et al., 2009).

Mutant SOD1 causes astrogliosis, activation, infiltration and the inflammatory response of microglia, (increased production of nitric oxide and toxic cytokines), leading to further damage to motor neurons and accelerated disease progression (Polazzi and Monti, 2010).

1.3.3 GLIAL CELL INVOLVEMENT IN ALS

Involvement of microglia in ALS was postulated in the early 90s in *in vivo* models of the disease, by studying microglial activation in the brain and spinal cord of patients. Activation of microglia correlates with disease progression, leading to the idea that immuno-inflammatory mechanisms may contribute to ALS. Neuro-inflammation in ALS is also indicated by the fact that anti-inflammatory drugs could exert protective effects in mouse models of ALS. However, the role of microglia in neuro-inflammation and whether microglial activation is a cause or a consequence of the degeneration of motor neurons has not been fully clarified. Metabolism of nitric oxide (NO) may provide a link between microglial activation and oxidative damage to motor neurons. Mutant SOD1 is able to catalyze the production of reactive oxygen species such as peroxynitrite, which promote the expression of inducible nitric

oxide synthase (iNOS) (Li et al., 2005). iNOS converts arginine into citrulline and produces NO. Production of NO may play an important role in the transition from M2 to M1 phenotype and consequently in neuronal toxicity (Lewis et al., 2014).

Astrocytes are the most abundant cells in the adult nervous system. In mouse models of ALS, astrocyte degeneration is due to the interaction of glutamate with one of its receptors, GluR2, which is less expressed than in control conditions. The GluR2 subunit is impermeable to Ca^{2+} and thus protects motor neurons from excitotoxicity. Mutant SOD1 expressed in
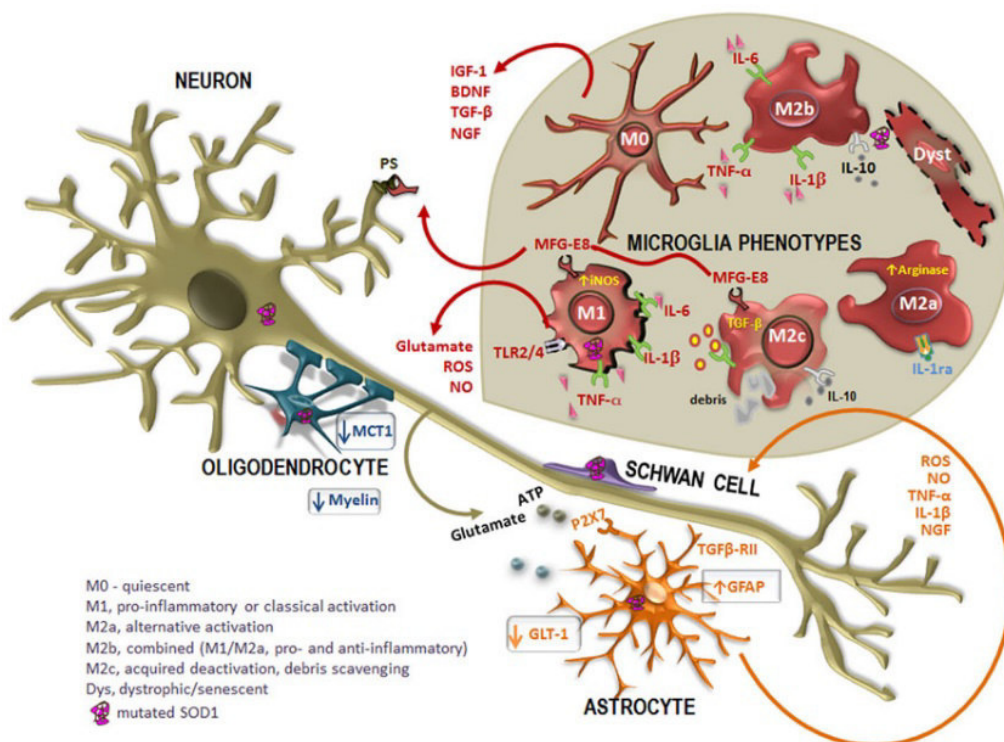
astrocytes alters the impermeable property of the receptor, making motor neurons vulnerable to excitotoxicity (Ilieva et al., 2009). Pharmacological blockade of the glutamate receptor slows the degeneration of astrocytes, delays the onset of symptoms and improves life expectancy. Thus the GluR2 glutamate receptor may be investigated as a therapeutic target. Although the expression of mutant SOD1 confined to astrocytes is not sufficient for disease development, selective reduction of mutant SOD1 in astrocytes slows down disease progression and the consequent activation of microglia, demonstrating the functional cross-talk between astrocytes and microglia. (Nagai et al., 2007).

Co-cultures between glia and neurons underline the role of glia in the onset and progression of ALS confirming the "non-cell autonomous" hypothesis for neurodegeneration in ALS (Wang et al., 2009).

1.4 ALPHA-SYNUCLEIN (α-syn)

SNCA is the gene encoding for α-synuclein. It is also called PARK1 and PARK4 and was mapped in 1997 on the long arm of chromosome 4 by US researchers who studied a famous Italian family, "Contursi family". (Polymeropoulos et al., 1997). It is composed by 7 exons of which 5 are encoding (Norris et al., 2004). α-synuclein is a ubiquitous protein belonging to a family that also includes β- and γ –synucleins. It is mainly expressed in the brain but also in the myocardium, connective tissue, spleen, prostate, thyroid, pancreas and in platelets. In the brain, alpha-synculein represents 0.5-1.0% of all cytosolic proteins and it shows both cytosolic and nuclear localization in neuronal cells and is primarily located at the presynaptic end (Iwai et al., 1995). α-synuclein is composed
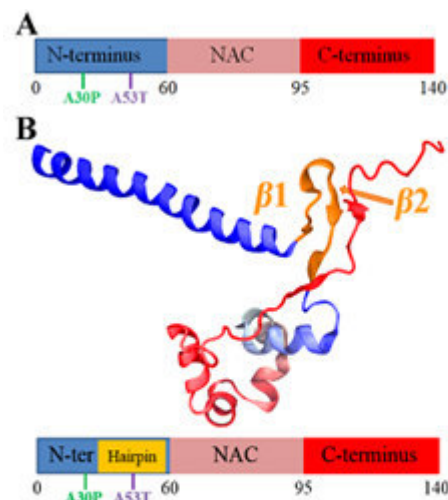
Transient β-hairpin formation in α-synuclein monomer revealed by coarse-grained molecular dynamics simulation. J Chem Phys. 2015.

of 140 amino acids and three distinct regions: an N-terminal amphipathic region, with a short conserved sequence and α-helix structures (1-60), a hydrophobic intermediate region (NAC) containing the non-amyloid β component (61-95) and a C-terminal region, characterized by long and variable sequences of serine and tyrosine residues involved in the interaction with other proteins (96-140) (Venda et al., 2010). Binding with phospholipids induces changes in the N-terminal region. The protein modifies its secondary structure from about 3% to over 70% of α-helices. The NAC domain promotes the formation of β sheets (Serpell et al., 2000; El-Agnaf and Irvine, 2000). The N-

terminus region and the NAC domain contain a binding domain responsible for the association between α-synuclein and the cell membrane (Eliezer et al., 2001). Alpha-synuclein protein produced by recombination or through purification does not show an orderly secondary or tertiary structure, however, its N-terminal region assumes an α-helix conformation upon binding with phospholipidic vesicles (Vekrellis et al., 2004).

Although the physiological function of α-synuclein has not yet been clarified, its interaction with synaptic vesicles has been shown through electron microscopy. It is generally associated to the pre-synaptic membrane, where it stabilizes the curvature of the bilayer and is involved in the maintenance of membrane integrity during the release of neurotransmitters (Eliezer et al., 2001) and in vesicle traffic and fusion during signal transmission at the synaptic level (Auluck et al., 2010; Iwai et al., 1995). Studies on α-synuclein knockout mices how that despite not being essential for the normal development of the nervous system, α-synuclein is involved in long-term regulation and in the maintenance of presynaptic function (Chandra et al., 2004) and it is necessary for the genesis, location and maintenance of the presynaptic reserve pool (Cabin D.E. et al., 2002). α-synuclein is transmitted from neuron to neuron and to astrocytes through endocytosis and inclusion bodies (Lee et al., 2010; Rappold and Tieu, 2010), but whether it can also be released by glial cells has not been yet clarified. α–synuclein has a predominantly cytosolic distribution but its ability to adhere to cell membranes allow it to display other cellular locations (Nakamura et al., 2011; Kamp et al., 2010). The presence of α-synuclein in mitochondria has been reported: some studies detected α-synuclein presence at the level of the outer mitochondrial membrane (Nakamura et al., 2011; Zhang et al., 2008) and the inner mitochondrial membrane in PD patients (Devi et al., 2008; Martin et al., 2006). Overexpression of α-synuclein interferes with complex I (Nakamura et al., 2011; Chinta et al., 2010; Loeb et al. 2010; Liu et al., 2009) and complex IV (Martin et al., 2006) activity of the mitochondrial respiratory chain and this leads to an increased production of reactive oxygen species and nitric oxide, resulting in oxidative stress and the release of cytochrome c (Parihar et al., 2009).

37

1.4.1 PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. PD affects more than 1% of the population aged 55 years and more than 3% of the population aged 75 years (Pankratz et al., 2009). There are also early-onset cases, which are found in 5-10% of patients, whose initial symptoms appear between ages 20 and 50. Much more rarely, juvenile-onset forms (EOPD) can be identified, whose symptoms appear before age 20 (Wood-Kaczmar et al., 2006). Only 5% of cases are inherited; the remaining 95% of cases are sporadic idiopathic and the causes are still not clear, although the main risk factor appears to be aging. PD was described for the first time in 1817. The English physician James Parkinson described the clinical history of 6 patients affected by a neurological disease, which he named "shaking palsy".

Characteristic hallmarks of PD are the death of nigral dopaminergic neurons projecting from the substantia nigra pars compacta and the presence in these neurons of cytoplasmic inclusions called "Lewy bodies", whose main component is represented by α-synuclein (Steiner et al., 2011; Kahle, 2008). The first gene related to the onset of PD was SNCA, encoding for α-synuclein (Polymeropoulos et al, 1997).

The monogenic forms of PD can be divided in autosomal dominant and autosomal recessive forms. Regarding the autosomal recessive forms, mutations in the parkin (PINK1) and protein deglycase (DJ1) genes are the most relevant clinical aspect (Houlden and Singleton, 2012; Zimprich et al., 2011). Regarding the autosomal dominant forms of PD, three are the genes involved; LRRK2, VPS35 and α-synuclein. Some point mutations (A53T, A30P and E46K) in α-synuclein gene are related to the onset of an autosomal dominant juvenile form of PD (Singleton et al., 2013; Zarranz et al., 2004; Polymeropoulos et al., 1997), while multiplication of the entire locus seems to lead to a more severe early onset of the disease depending on the number gene copies (Fuchs et al., 2007; Singleton et al., 2003).

Synucleopaties models (Maries et al., 2003).
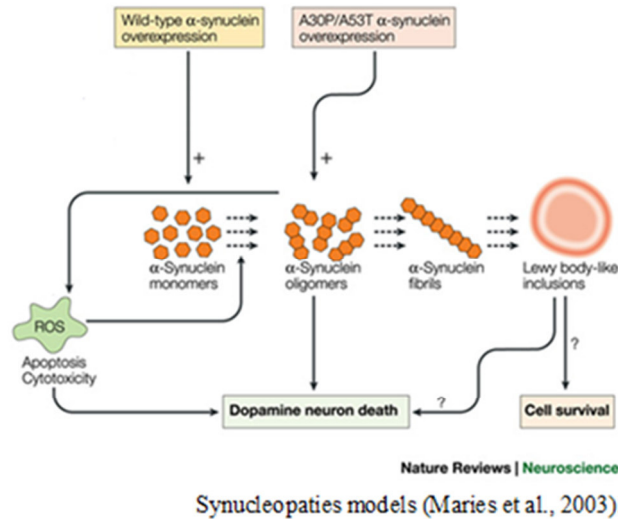
While the causes responsible for neuronal death remain largely unknown, the most likely hypotheses are pathogenic factors common to many neurodegenerative diseases, such as oxidative stress, apoptosis, mitochondrial dysfunction, neuroinflammation and dysfunction of protein degradation mechanisms (Bové and Perier, 2012). In this complex scenario, it is plausible that both genetic and environmental factors may contribute to PD development. Clinical description of PD is based on motor symptoms: bradykinesia, rigidity, resting tremor and postural instability (Schrag and Quinn, 2000). Recently, non-motor symptoms related to the disease have been discovered (Martinez-Martin et al., 2011; O'Sullivan et al., 2008), anxiety, depression, psychosis and impulse control disorder, sleep disturbances, cognitive and sensory disorders, cardiovascular, gastrointestinal and genitourinary disorders (Dissanayaka et al., 2010; Weintraub et al., 2010). Moreover, dementia occurs in 20% of cases (Pankratz et al., 2009). Currently there are no treatments available to counteract the disease, only therapies that slow down progression. The most common pharmacological approach is the treatment of PD patients with levodopa, a dopamine precursor, able to cross the blood-brain barrier where it is converted in dopamine. Therapy with levodopa improves the life of patients, but after few years of treatment, they show side effects characterized by alternative periods of preserved motor capability and periods of akinesia or tremor. Because PD causes loss of at least 70% of neurons in the nervous system, the aim of new

pharmacological therapeutic trends is to find neuroprotective and neurotrophic drugs.

1.4.2 MUTANT α-syn IN PARKINSON'S DISEASE

Interest in α-synuclein has increased due to its direct involvement in many neurodegenerative diseases. Its pathogenicity, in fact, is related to its propensity to aggregate and to be the main component of amyloid plaques in Alzheimer's patients (Campion et al. 1995).
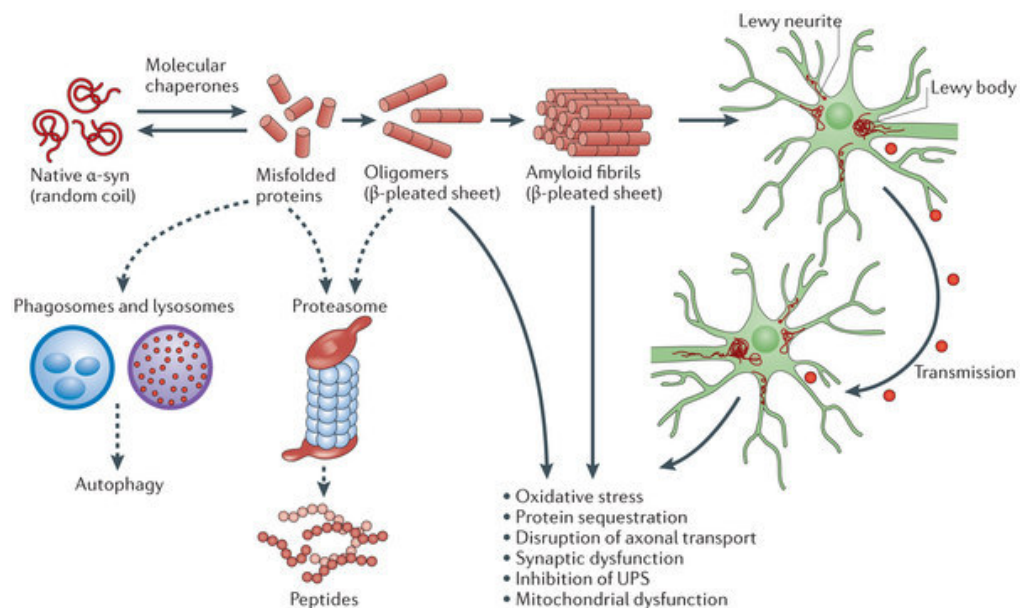
α-syn toxicity model (Irwin et al., 2013).

In 1997 a mutation on chromosome 4 was identified in PD patients and one year later it was shown to be the missense mutation A53T in the α-synuclein gene (Polymeropoulos et al. 1997). α-synuclein fibrils are the main component of Lewy bodies in PD patients, but they are also found in other types of aggregates, Lewy neurites or oligodendroglial inclusions, that characterize diseases indicated as "synucleinopathies" (Steiner et al., 2011; Kahle, 2008;). In this group of pathologies, we find dementia with Lewy bodies (DLB), a variant of Alzheimer's disease (LBVAD), Huntington's

disease, fronto-temporal dementia, progressive supranuclear palsy, Down syndrome, multiple system atrophy (MSA) and neurodegeneration with iron accumulation type 1 (NBIA-1) (Jellinger, 2003). The molecular mechanisms linking α-synuclein to the pathogenesis of these diseases are not yet fully understood. In the past years, it was believed that the presence of mutant α-synuclein or its overexpression could promote the formation of toxic protein aggregates. Recently, it has been demonstrated that in case of special types of injuries, α-synuclein has a protective role. According to the current state of the art, the protective or toxic effect of α-synuclein depends on its level of expression in the cell: α-synuclein in physiological conditions would have a protective function, while its accumulation and aggregation into oligomers or fibrils would have a detrimental effect for cells (Irwin et al., 2013; Vekrellis et al., 2004).

Missense mutations in the gene (A53T, A30P), duplication or triplication of the wild-type form of α-synuclein are neurotoxic and responsible for familial forms of PD (Mosharov et al., 2006). In solution, A53T and A30P mutations increase the rate of oligomerization of α-synuclein and the formation of β sheets (Uversky and Fink, 2002); the mutation rate of the fibrils is increased by the A53T mutation and reduced by the A30P mutation (Conway et al., 2000) and α-synuclein overexpression causes the formation of aggregates in many cell lines, while in mouse models, the formation of non fibrillar inclusions in the same brain regions as Lewy bodies. Mice also display damage in the nigrostriatal dopaminergic system and movement deficits (Stefanova et al., 2001). In an α-synuclepin transgenic model of Drosophila melanogaster, neuronal loss and formation of inclusions similar to Lewy bodies from patients were observed (Feany and Bender, 2000). Accumulation of α-synuclein causes the blockade of vesicular trafficking from the endoplasmic reticulum to the Golgi apparatus (Cooper et al., 2006). Moreover, α-synuclein has been shown to be involved in the modulation of mitocondrial functions (Stichel et al., 2007; Martin et al., 2006).

Lastly, α-synuclein activity is related to the presence of dopamine (DA). Catecholamines, in particular dopamine, react with α-synuclein in order to stabilize the protofibrils and promote their aggregation (Conway et al., 2001).

Also other conditions seem to facilitate α-synuclein aggregation: contact with toxic substances, such as paraquat and rotenone, which act through the inactivation of mitochondrial activity, oxidative stress and even aging. In fact, aged cells are no longer able to remove α-synuclein, therefore its levels increase in the cytoplasm and accumulate pathologically (Li et al., 2004). According to its conformational and functional state, α-synuclein can be degraded by the ubiquitin-proteasome system or through autophagy. In the past, it was believed that soluble forms were degraded by the ubiquitin-proteasome system, while aggregates through autophagy. Subsequent studies have shown that even the soluble forms of α-synuclein can reach the lysosomal compartment to be degraded (Jackson-Lewis et al., 2000). The ubiquitin-proteasome system seems unable to remove α-synuclein mutant forms and fibrils that accumulate in aggregates (Singleton et al., 2003).

1.4.3   GLIAL   CELL   INVOLVEMENT   IN PARKINSON'S DISEASE

Microglia seems to play an important role in PD given that the substantia nigra pars compacta is the brain region with the highest density of microglia and in PD patients, the presence of reactive microglia which stimulates the production of free radicals, resulting in neuronal oxidative damage has been reported. *In vitro*, overexpression of wild-type or mutant human α-synuclein (A53T and A30P) in the BV2 microglial cell line, drives microglial cells towards the reactive phenotype characterized by the increase of arachidonic acid metabolism, cytokine and interleukin secretion, reactive oxygen species, tumor necrosis factor α (TNF-α) and nitric oxide (NO) production (Rojanathammanee et al. 2011).

In addition to microglia, astrocytes also confer neuroprotection, both through the removal of toxic molecules from the extracellular space and through the release of trophic factors and antioxidant molecules. However, in pathological conditions, astrocytes release proinflammatory cytokines and other toxic molecules that are harmful to dopaminergic neurons. Astrocytes density is low in the substantia nigra pars compacta, which is the most affected

region, while it is high in other less affected areas in the disease, as in the ventral tegmental area. Therefore Damier and colleagues hypothesized that neurons in the substantia nigra pars compacta might be more vulnerable to illness because of the lack of neuroprotective glial cells (Hirsch and Hunot S, 2009; Damier et al., 1993). Astrocytes confer neuroprotection to dopaminergic neurons via lysosomes eliminating the toxic extracellular excess of α-synuclein. Lee and colleagues reported that α-synuclein is transmitted from neuron to neuron and from neuron to astrocyte through endocytosis and inclusion bodies (Lee et al., 2010) and this property led them to suggest that α-synuclein may have prion-like properties (Rappold et al., 2010).

2. AIMS

Previous results from our laboratory clearly demonstrated that microglial cells exert a neuroprotective role through the release of soluble factors (Polazzi et al., 2001, 2009, 2013, 2015; Polazzi & Monti, 2010). In order to clarify this neuroprotective effect both in physiological and pathological conditions, a firm aim of this thesis was to check whether i) proteins related to neurological diseases were released by glial cells, ii) their release could also be mediated by vesicles, iii) they could be involved in glial activation and therefore neuroinflammation.

To perform these experiments we used *in vitro* models of glial cells: primary cultures of rat astrocytes and microglia, as well as immortalized mouse astrocytes and microglia cell lines (respectively IMA2.1 and N9).

We focused our attention on three proteins: PNP, SOD1 and alpha-synuclein.

The interest on these proteins derived from the fact that they are all involved in brain neuropathology, since mutations in the gene codifying for PNP induce PNP deficiency, a neurodevelopmental disorder characterized by immunodeficiency, while SOD1 and alpha-synuclein are both involved in neurodegenerative diseases, amyotrophic lateral sclerosis and Parkison's disease respectively, both characterized by the presence of protein aggregates, neurodegeneration and neuroinflammation.

Because in all these neurological diseases there is a strong involvement of immune cells, we focused on the role of glial cell secretion, since most studies focused on neurons only.

Neurodegenerative diseases, stroke, trauma and hypoxia affect neuronal survival indirectly by triggering neuroinflammation. Microglia are then activated in response to insults acquiring a phagocytic phenotype and releasing inflammatory mediators, cytokines and chemokines. The acute neuroinflammatory response seems to be beneficial to the CNS, since it is an attempt to reduce injury and restore damaged tissues.

Because glial cells play a crucial role in brain pathophysiology, mainly following their activation, we assessed whether the release of these proteins

could be influenced by typical inflammatory stimuli, such as lipopolysaccharide (LPS), interferon-γ (IFN-γ) and ATP.

Since it is known that protein release in glial cells could occur through the lysosomal secretory pathway (Dubyak, 2012; Takenouchi, 2009) and since glial secretion through extracellular vesicles (EVs) has acquired increased importance in physiological glial-neuron communication, we also assessed whether PNP, SOD1 and alpha-synculein could be released by glial cells through this pathway.

Considering previous studies from our laboratory on the neuroprotective role of microglial cells against neuronal damage (Polazzi et al., 2013), the second aim of this project was to study the role that proteins secreted by glial cells may have on pathological conditions.

Polazzi et al., demonstrated a neuroprotective role of microglia exerted by low molecular weight secreted proteins. SOD1 was identified as one of these molecules, constitutively produced, released and accumulated in conditioned medium (MCM). However, mutations in SOD1 are some of the main hallmarks observed in patients affected by familial Amyotrophic Latera Sclerosis (ALS) and glial cells appeared to be crucial for the initiation and progression of disease, since a non-cell autonomous mechanism for ALS neurodegeneration has been postulated (Ilieva et al, 2009).

Mutant SOD1 has been shown to cause a reduction in glial cell physiological function, activation of microglial cells characterized by an elevated production of cytotoxic molecules such as ROS, inflammatory molecules including iNOS, pro-inflammatory cytokines and failure of astrocyte glutamate uptake, thus suggesting that motor neuron degeneration could be triggered by glial cell dysfunction (Weydt et al., 2004; Hensley et al., 2006; Liao et al., 2012; Frakes et al., 2014).

In agreement with these evidences, we overexpressed in rat primary cultures of microglia and astrocytes wild-type SOD1 and fALS-linked SOD1 mutants (G93A and A4V), to compare the effect of different mutations on glial cell activation and microglial phenotype.

Glutamate excitotoxicity is considered one of the main causes of neuronal death in ALS; in order to clarify the role of glial cells in ALS

neurodegeneration, we developed an *in vitro* model of primary neuron-microglia and neuron-astrocyte contact co-cultures to evaluate the effect of wild-type and mutant SOD1 overexpression in glial cells on neuronal survival and death after exposure to glutamate.

Furthermore, we carried on a parallel study on α-synuclein expression and by from glial cells as well as on the effect of wild-type or familial PD-linked mutant α-synuclein over-expression. So far, most studies have mainly investigated the role of α-synuclein in neurons, excluding non-neuronal cells, such as glial cells. Parkinson's disease (PD), however, involves the alteration of many other cell types besides neurons, modifying not only neuronal function, but also the cross-talk between different neurons and neighbouring cells (Appel et al., 2010). According to the "non-cell autonomous" hypothesis, we started a preliminary study on the expression and release of α-synuclein in physiopathologycal conditions. First, we assessed whether glial cells released α-synuclein and whether the release could be influenced by typical inflammatory stimuli, such as lipopolysaccharide (LPS) and ATP. α-synuclein is transmitted from neuron to neuron and from neuron to astrocyte through endocytosis and inclusion bodies (Rappold et al., 2010; Lee et al., 2010), however whether it could be released even by microglial cells is still unclear. We also performed experiments to verify the involvement of the lysosomal secretory pathway and vesicles in α-synuclein release.

The role of D-serine in the modulation of N-methyl-D-aspartate receptors (NMDAR) is known; D-serine is synthetized by astrocytes from L-serine. Glial cell activation stimulates the release of cell death mediators, such as glutamate, cytokines and reactive oxygen species (ROS). The increase in extracellular glutamate levels is considered one of the main causes of neuronal death (Van Damme et al., 2005; Bruijn et al., 2004). We assessed whether L-serine could mediate α-synuclein release.

α-synuclein is also involved in neurodegenerative diseases; its pathogenicity is related to its propensity to aggregate, as shown to be the main component of amyloid plaques in Alzheimer's patients (Campion et al. 1995).

Missense mutations in the α-synuclein gene (A53T, A30P), or duplication or triplication of the wild-type form are neurotoxic and responsible for familial forms of PD (Mosharov et al., 2006).

To this aim, we overexpressed in immortalized cell lines of microglia and astrocytes wild-type or familial PD-linked mutant α-synuclein (A53T and A30P), to compare the effect of different mutations on glial cell viability.

3. MATERIAL AND METHODS

3.1 PRIMARY RAT CULTURE OF MICROGLIA AND ASTROCYTES

Animals sacrificed for primary cell cultures were Wistar rats, bred with free access to food and water and kept in a light-dark 12/12 hour cycle (Levi et al., 1993). Experiments were conducted in accordance to European Community and Italian laws, and approved by the Ethical Committee for Animal Experimentation of the University of Bologna (Protocol No. 17-72-1212). In addition, University veterinarians periodically checked animal health.

In order to allow the cell adhesion, flasks were treated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA), a synthetic compound that enhances cell adhesion favoring growth and differentiation. The 1 mg/ml stock solution of poly-L-lysine (Sigma, 30,000 <MW <70,000) was resuspended in sterile distilled water and used at a concentration of 10 µg/ml. The entire surface of the flasks was covered with poly-L-lysine, which was removed after 30 minutes and flasks were left to dry. We obtained mixed cultures of glial cells from cerebral cortices of newborn rats. After 6/7 days in culture, we were able to obtain pure secondary cultures of microglia through mechanical detachment, while pure astrocyte cultures were obtained by trypsinization. The complete medium used to maintain cultured glial cells consisted of Basal Medium Eagle (BME, Life Technologies Ltd, Paisley, UK) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Life Technologies), 50 mg/ml gentamicin and 2-mM L-glutamine (Sigma-Aldrich).

The 5 solutions used for the mixed culture preparation were composed as follows:

SOLUTIONS		
Solution 1	KREBS 10X	15 ml
	MgSO4 3,82%	1,2 ml
	BSA	0,45 g
	H_2O	135 ml
Solution 2	Trypsin	9,38 mg
	Solution 1	37,5 ml
Solution 3	Solution 1	22,5 ml
	DNAse	5,4 mg
	Trypsin inhibitor	11,7 mg
	$MgSO_4$ 3,82%	225 µl
Solution 4	Solution 1	31,5 ml
	Solution 3	6 ml
Solution 5	$CaCl_2$ 1,2 %	22,5 µl
	$MgSO_4$ 3,82%	150 µl
	Solution 1	18,75 ml

KREBS STOCK SOLUTION 10X pH 7.4

NaCl (1.32 M)	38,6 g
KCl (0.05M)	1.85 g
Na_2HPO_4 + 12 H_2O (0.085M)	15,2g
$NaHPO_4+H_2O$ (0.21M)	1,45g
Glucose (0.1M)	9,9 g
Phenol red	50 mg
H_2O	500 ml

All reagents were from Sigma-Aldrich.

To prepare mixed primary cultures of glial cells, newborn Wistar rats (1 day) were sacrificed and cortices were immediately removed. From each animal we obtained of 5/6 mixed culture 75 cm^2 flasks on average. Meninges were removed by mechanical dissociation in solution 1, using sterile tweezers. 20 ml of solution 2 containing trypsin were added to the hemispheres in solution. In order to allow enzymatic dissociation, tissues were incubated in a water bath at 37 ° C for 15 minutes. At this point, solution 4 containing the trypsin inhibitor trypsin and DNAse were added to block the reaction. After a quick centrifuge (1000 rpm, 4 minutes) the supernatant was removed and the pellet was resuspended in 5 ml of solution 3 with a glass Pasteur pipette in order to mechanically obtain complete tissue dissociaion. After a last wash in solution 5, the pellet was resuspended in BME (Life Technologies)

supplemented with 10% FBS (Life Technologies), 50 mg/ml gentamicin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich), previously heated at 37 ° C and cells were plated in poly-L-lysine coated flasks (Sigma-Aldrich). Flasks were kept in a humidified incubator at 37 ° C with 5% CO_2. After 2 days, the medium was replaced with fresh medium to remove debris and non-adherent cells. After 7-8 days in culture, astrocytes were confluent and microglia grew on the astrocyte monolayer.

To obtain a pure secondary culture of microglia, microglia were detached by mechanically shaking the flasks, the cell culture medium was collected and centrifuged. The obtained pellet was resuspended in 10 ml of serum-free BME (Life Technologies), cells were counted with Neubauer counting chamber and plated at the density of $1.5x10^6$ or $2x10^6$ /35mm Ø dish in 2ml of serum-free BME (Life Technologies), depending on the experiment. Flasks were replaced with fresh complete BME (Life Technologies) and after 3-4 more days microglia was ready to be detached again.

To purify astrocyte cultures, 10-day-old primary mixed glial cultures were vigorously shaken to detach microglia and oligodendrocytes growing on top of the astrocyte monolayer. The remaining adherent cells were detached with 0.25% trypsin/EDTA (Life technologies), centrifuged and resuspended in fresh BME (Life Technologies) medium without serum and plated on poly-L-lysine-coated (Sigma-Aldrich) 35 mm Ø Petri dishes at a density of $0.5 x 10^6$ cells/1 ml medium/well.

3.2 PRIMARY RAT CULTURE OF CEREBELLAR GRANULE NEURONS (CGNs)

Cerebellar granule neurons (CGNs) obtained from 7-day old rats reach full differentiation after 7 days further in culture (7 DIV) (Gallo et al., 1987). Primary CGNs are the most-widely used model to study cellular and molecular mechanisms of survival and neuronal death, since it is an almost pure culture, with no contamination with glia or other types of neurons (Contestabile A. 2002). The culture medium used to maintain neurons was composed by BME (Life Technologies) supplemented with 10% heat-inactivated FBS (Life

Technologies), 50 mg/ml gentamicin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 25mM KCl (Sigma-Aldrich).

In order to prepare this neuronal culture, the following 5 solutions were used:

SOLUTIONS		
Solution 1	KREBS 10X	25 ml
	MgSO4 3,82%	2 ml
	BSA	0,75 g
	H_2O	up to 250 ml
Solution 2	Trypsin	12.5 mg
	Solution 1	up to ml
Solution 3	Solution 1	Up to 25 ml
	DNAse	3.12 mg
	Trypsin inhibitor	13 mg
	$MgSO_4$ 3,82%	250 µl
Solution 4	Solution 1	8 ml
	Solution 3	42 ml
Solution 5	$CaCl_2$ 1,2 %	320 µl
	$MgSO_4$ 3,82%	320 µl
	Solution 1	Up to 40 ml

All reagents were from Sigma-Aldrich.

To obtain a primary culture of cerebellar granule neurons, we collected cerebella from 7-day old rats. After meninges were removed, the cerebellar tissue was collected in a 50 ml falcon tube with solution 2 containing trypsin and incubated in a water bath at 37 ° C for 15 minutes in order to enhance the enzymatic dissociation of the tissue. At this point, trypsin activity was blocked by adding solution 4 containing trypsin inhibitor and DNAse (Sigma-Aldrich), to degrade DNA that may accumulate after cell disruption. After a quick centrifuge, 4 minutes at 1000 rpm,the pellet was mechanically dissociated in solution 3. Then, after a wash in solution 5 and the last centrifuge, 7 minutea at 1000 rpm, the cell pellet was resuspended in complete BME (Life Technologies), cells were counted and plated at the density of 2.4x10^6 cells in 2 ml of medium in 35 mm Ø Petri dishes previously coated with poly-L-lysine (Sigma-Aldrich) as previously described. After 16h in a humidified incubator at 37 ° C, 5% CO_2, 10 mM cytosine arabinoside (Sigma-Aldrich) was added to cell cultures to reduce the proliferation of non-neuronal cells and obtain pure

neuronal cultures. After 7 days in vitro (7-DIV), cerebellar granule neurons were fully differentiated and ready to be used.

3.3 IMA 2.1 AND N9 IMMORTALIZED CELLS LINES

In order to use immortalized cell line cultures, sterile instruments and solutions were used under a laminar flow hood. To increase cell adhesion, Petri dishes were pre-coated with poly-L-lysine (Sigma-Aldrich), as previously described.

IMA 2.1 cells are an immortalized murine astrocyte cell line, while N9 cells are an immortalized murine microglia cell line.

IMA 2.1 and N9 cell lines were kept in culture in 10 cm Ø Petri dishes with 9 ml of Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% FBS, 1% Penicillin/Streptomycin solution and 2 mM L-glutamine. For subsequent experiments, cells were trypsinized and plated at an initial density of 5×10^5 cells/60 mm diameter Petri dish and 2×10^5 cells /35 mm diameter dish for 24 hours in complete DMEM medium. 60 mm diameter Petri dishes were used for Western blot analysis and nitrite detection, while 35 mm diameter Petri dishes were used for immunocytochemistry and Hoechst staining.

3.4 CULTURE TREATMENTS

3.4.1 TREATMENT WITH LPS

In order to activate microglial cells, one of the first methods described in the literature was treatment with the bacterial endotoxin lipopolysaccharide (LPS) (Hetier et al., 1988). *In vitro* activation of microglia with LPS mimics the glial activation that occurs *in vivo*, as a result of an acute inflammatory stimuli, but also chronic microglial activation due to neurodegenerative diseases (Petrova et al., 1999; Pahan et al., 1997; Nishiya et al. 1995). LPS

treatment is known to increase the production and release of pro-inflammatory cytokines and chemokines (Qin et al., 2004; Kong et al., 1997; Lieberman et al., 1989), as well as to induce the up-regulation of nitric oxide synthase (iNOS) and the consequent increase of nitric oxide (NO) secretion (Pahan et al., 1997; Boje and Arora, 1992) mediated by the TLR4 receptor (Takeda et al., 2003). Primary microglia and astrocytes, as well as immortalized N9 and IMA 2.1 cell lines were plated in BME (microglia and astrocytes) or DMEM (N9 and IMA2.1) medium supplemented with 2 mM glutamine and 1% penicillin/streptomycin, without serum at previously described denisities and stimulated for 24 hours with increasing concentrations of LPS [1; 10; 100; 1000 ng/ml].

3.4.2 TREATMENT WITH INTERFERON- γ (IFN- γ)

In parallel to LPS stimulation, primary microglia and astrocytes were also treated with interferon-γ (IFN-γ). IFN-γ is produced by activated T lymphocytes and natural killer cells; it modulates immunological responses and stimulates the production of reactive nitrogen and oxygen species and cytokines/chemokines in microglia (Schroder et al., 2017). Primary microglia, astrocytes and neurons were plated in BME medium without serum, as previously described, and stimulated for 24 hours with increasing concentrations of IFN-γ [1; 10; 100 ng/ml].

3.4.3 TREATMENT WITH ADENOSINE TRIPHOSPHATE (ATP) AND 2'-3'-O-(4-BENZOYLBENZOYL)-ATP (BZ-ATP)

Adenosine triphosphate (ATP) and its stable form 2'-3'-O-(4-benzoylbenzoyl)- ATP (Bz-ATP) are stimuli both able to activate P2X7 receptors expressed by macrophages and microglia. Activation of microglia by ATP induces the formation of membrane permeable pores, stimulates monocyte and macrophage lysosomal secretion, induces the activation of intracellular signals including the influx of calcium which in turn stimulates the

activation of the MAP kinase pathway and eventually the activated state of microglia. ATP-mediated activation through the activation of P2X7 receptors appears to be not chronic, less aggressive than the activation mediated by LPS, and mainly leading to an apoptotic condition (Takato et al., 2009).

Primary cultures of glial cells were stimulated for 1 hour with increasing concentrations of ATP [0.5, 1 mM] or Bz-ATP [125, 250 µM].

Oxidized ATP (ox ATP) is an inhibitor of P2X7 receptors, known to inhibit T- cell proliferation and interfere with the innate immune response (Lang et al., 2010). Suramin is a non-selective antagonist of P2 receptors and, in a similar way to ox ATP, it could inhibit the immune response, though it also has anti-inflammatory proprieties. Microglia and astrocytes were pre-treated with 300µM ox ATP or 50 µM suramin for 1 hour and then treated with ATP or Bz-ATP.

N9 and IMA2.1 cells were stimulated for 4 hours with increased concentrations of ATP [100, 500 µM]. Cells were collected in lysis buffer (1% SDS, 50 mM Tris HCl pH 7.4, 1 mM EDTA, protease inhibitor cocktail 10 µl/ ml) and stored at -80°C for western blot analysis. An equal volume of conditioned medium (500µl) was collected and concentrated by using Microcon-YM-3 (EMD Millipore Corporation, Billerica, MA, USA), resuspended in 15 µl of Loading buffer 4 X (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, 1 M DTT 1:5) and stored at -20 ° C for western blot analysis. In parallel, primary cultures of microglia and astrocytes were treated with 500 µM ATP and fixed with 4% paraformaldehyde (PFA) for immunocytochemistry assays.

3.4.5 L-SERINE TREATMENT

D-serine is a neuromodulator of N-methyl-D-aspartate receptors (NMDAR), able to allow the opening of these receptors by glutamate. The NMDA receptor has a binding site for glycine, but D-serine is an endogenous agonist stronger than glycine (Panatier et al., 2006). D-serine is synthesized in astrocytes from L-serine thanks to the enzyme serine racemase (SR), expressed mainly in glial cells (Wolosker et al., 1999) and upregulated by glial activation

(Wu and Barger, 2004). The SR enzyme catalyzes the elimination of water from both L-serine and D-serine to form pyruvate (Foltyn V.N. et al., 2004).

We treated IMA 2.1 and N9 cell lines with different concentrations of L-Serine (Sigma-Aldrich). IMA 2.1 and N9 cells were plated as previously described and treated for 24 hours with increasing concentrations of L-serine in DMEM without serum [0,10,50,100 μM].

3.4.6 TREATMENT WITH THREALOSE

We treated primary cultures of microglia and astrocytes overexpressing wild-type and mutant SOD1 with 5mM trehalose (Sigma-Aldrich), which is able to induce autophagy, for 48 hours in serum-free BME. $2x10^6$ microglial cells and $5 \ x10^5$ astrocytes were plated in 35 mm diameter Petri dishes in serum free BME, transfected with SOD1 plasmids as described in the following paragraphs and treated with thehalose.

3.5 GLIAL CELL TRANSFECTION

3.5.1 PURIFICATION OF PLASMID DNA

In order to amplify the plasmids used for cell transfection (pcDNA3.1 (+)), competent bacteria were used (E. coli, DH-5α strain). Bacteria were first subject to thermal shock, which allows an increase in the permeability of the plasma membrane and the consequent entry of the plasmids within the bacteria. Starting from a "pre-inoculum" of transformed bacteria selected by the presence of plasmids with antibiotic resistance, 1 ml of pre-inoculum was added to 50 ml Lurina-Bertani broth (LB) with ampicillin at a final concentration of 100 μg/ml and incubated at 37 ° C on an orbital shaker at 250 rpm overnight.

The next day, the bacterial suspension was centrifuged at 4000 rpm (USA LE G DEL PROTOCOLLO) for 15 minutes at 4 ° C. To purify plasmid DNA, the NucleoBond XTra kit Midi / Maxi (Macherey-Nagel) was used. The kit consists of resuspension buffer RES (50 mM Tris-HCl, 10 mM EDTA, 100

mg/ ml RNase A, pH 8.0), bacterial Lysis Buffer (200 mM NaOH, 1% SDS) added to the cell pellet and gently mixed by inversion, allowing the lysis of the bacterial cells for 5 minutes. At this point, equilibration EQU Buffer (100 mM Tris, 15% ethanol, 900 mM KCl, Triton X-100 0.15% adjusted to pH 6.3 with H_3PO_4) was used to balance the purification columns and inserted paper filters. Neutralization Buffer NEU (KAC 2.8 M, pH 5.1) was added to the cell lysate, mixed by inversion, and loaded onto the previously equilibrated columns with paper filters. The paper filters were again washed with buffer EQU before being removed and the columns were washed with washing Buffer WASH (100 mM Tris, 15% ethanol, 900 mM KCl, Triton X-100 0.15% adjusted to pH 6.3 with H_3PO_4). Plasmid DNA was bound by the column resin and eluted by adding elution Buffer ELU (100 mM Tris, 15% ethanol, 1 M KCl, adjusted to pH 8.5 with H_3PO_4) and 2-isopropanol was added to the eluate at room temperature. Plasmid DNA was centrifuged at 15000 g for 30 minutes at 4 ° C and the DNA pellet was washed again with 70% ethanol. The pellet was left to dry at room temperature, before being resuspended in sterile deionized H2O. Plasmid concentration and purity were then determined by using a UV Nanodrop spectrophotometer (Thermo scientific, NanoDrop Products, Wilmington, DE, USA). pcDNA3.1 plasmids encoding for human WT SOD1, G93A SOD1 and A4V SOD1, were kindly provided by Prof. Angelo Poletti, University of Milan, Italy (Sau et al., 2007). pcDNA3.1(+) plasmids encoding for WT α-synuclein, A30P α-synuclein A30P and A53T α-synuclein, were kindly provided by Dr. Nelson Cole (Department of Health & Human Services. National Institute of Health, Maryland, USA).

3.5.2 TRANSFECTION OF GLIAL PRIMARY CULTURES

To transfect primary microglia and astrocytes with SOD1 plasmids, we used Lipofectamine® 2000 (Life Technologies). Lipofectamine is a cationic lipid forming liposomes (lipid vesicles containing exogenous DNA) which

allows the formation of a complex containing negatively charged nucleic acids able to fuse with the cell membrane.

Microglial cells were plated at a density of 2×10^6 cells/2ml serum-free BME (Life Technologies) /35-mm Ø Petri dishes coated with poly-L-lysine (Sigma-Aldrich). Astrocytes were plated at a density of 5×10^5 cells/1ml serum-free BME/35-mm Ø Petri dishes coated with poly-L-lysine (Sigma-Aldrich). Cells were transiently transfected with a pEGFP empty vector, as transfection control, and pEGFP encoding for wild-type or mutant (G93A and A4V) human SOD1. According to the manufacturer's protocol, we used 1,25 µg of pDNA and 2,5 µl of Lipofectamine (Life Technologies) 2000 for 1×10^6 cells in Opti-MEM/serum-free BME (Life Technologies) without antibiotics for 2 hours; the medium was then replaced with serum-free BME and cells were incubated for 24 hours. Conditioned media were collected and stored at -20°C for western blot analysis and nitric oxide detection assay.

3.5.3 TRANSFECTION OF GLIAL CELL LINES

Transient transfection with empty vector, WT α-synuclein, or mutant α-synuclein (A30P and A53T) of glial cells lines was performed with polyethyleneimine (PEI) (Boussif O. et al. 1995), a polymer that at neutral pH has a high cationic charge and it is able to bind DNA, entering into the cell by endocytosis.

IMA 2.1 and N9 cells were plated in 6 cm Ø Petri dishes for western blot analysis and in 35 mm Ø Petri dishes for immunocytochemistry. The final transfection volume was 1 ml per 6 cm Ø Petri dish using 7 micrograms of pDNA and 500µl for 35 mm Ø Petri dishes using 4 micrograms of each pDNA. Each pDNA and PEI were equilibrated in 150 mM NaCl in order to have 1/20 of the final transfection volume for each mix. Once equilibrated, the PEI mix was added to the pDNA mix and after 15 minutes, the PEI-pDNA mix was added to cells plated in serum free DMEM.

3.6 CELLS AND CONDITIONED MEDIA

Glial cells or immortalized cell lines plated at the previous described densities, and conditioned media, were collected 2, 4, 24 or 48 hours after the respective treatments. Cells were collected in lysis buffer (1% SDS, 50 mM Tris HCl pH 7.4, 1 mM EDTA, protease inhibitor cocktail 10 µl/ ml) and stored at -80°C for western blot analysis. 500µl of conditioned medium were collected and concentrated by using Microcon-YM-3 filters (EMD Millipore Corporation, Billerica, MA, USA), resuspended in 15 µl of 4 X Loading buffer (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, DTT 1:5 of the total volume) and stored at -20 ° C for western blot analysis.

3.7 PURIFICATION OF EXTRACELLULAR VESICLES FROM CONDITIONED MEDIUM

2h serum-free medium was collected from cultures of primary microglia (1.8×10^6 cells/ 35 mm Ø dish), primary astrocytes (6×10^5 cells/ 35 mm Ø dish) or from IMA2.1 and N9 cell lines (5×10^5 cells/well in 60 mm Ø dishes) treated or not with 500 µM ATP, centrifuged for 10 minutes at 1500 rpm at room temperature, filtered through 0.22 µm filters and immediately ultracentrifuged for 2 h at 100,000 g, 4°C. Aliquots of conditioned medium and of the supernatant (500 µl) obtained after ultra-centrifugation were concentrated by using Microcon-YM-3 (Millipore, Billerica, Mass., USA) and then resuspended in 20 µl Loading buffer 4 X (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, DTT 1:5 of the total volume) as well as pellets obtained after ultracentrifugation and stored at -20 ° C for western blot analysis.

3.8 CO- CULTURE OF CEREBELLAR GRANULE NEURONS (CGNs) with PRIMARY MICROGLIA OR ASTROCYTES OVEREXPRESSING wt/ mutSOD1

Co-cultures are one of the best *in vitro* systems to study cell interactions. There are different co-cultures systems; neuronal exposure to conditioned medium, trans-well systems or direct contact co-cultures. In the experiments reported in this thesis, direct contact co-cultures between cerebellar granule neurons and glial cells (microglia or astrocytes) were used.

Co-cultures were set up by plating microglia or astrocytes (in serum free BME KCl 25mM), previously transfected in suspension with wt or mutSOD1 plasmids on top of fully differentiated neurons (7-DIV). $2x10^6$ microglial cells or $5x10^5$ astrocytes overexpressing SOD1 were plated on top of $2.4x10^6$ differentiated cerebellar granule neurons. Both types of co-cultures were subjected to treatment with 100 mM glutamate for 24h, which mimics excitotoxicity, or with trehalose or with both trehalose and glutamate. After 24h, co-cultures were fixed with 4% paraformaldehyde in PBS and nuclei were stained with Hoechst 33258 (2µg/ml; Sigma-Aldrich) to quantify neuronal damage as described in the immunocytochemiststy paragraph.

3.9 IMMUNOCYTOCHEMISTRY

Immunocytochemistry is a highly specific method to define the intracellular localization of proteins. The technique is based on the principle of antigen-antibody conjugation and a detection system that makes visible the conjugated antibodies with a fluorescent microscope. There are direct or indirect methods, the latter involving the use of a primary antibody specifically raised against the molecule of interest and a secondary antibody conjugated to a fluorescent substance.

$2x10^6$ microglial cells or $5x10^5$ astrocytes plated on 35 mm Ø Petri dishes in serum free BME, with or without ATP stimulation were used to assay PNP localization; $2x10^6$ microglial cells or $5x10^5$ astrocytes plated on 35 mm

Petri dishes Ø in serum free BME overexpressing wt and mutSOD1 were used to assay SOD1 localization and autophagy in physio-pathological conditions; 2.5×10^5 IMA2.1 or N9 cells plated in 35 mm Ø Petri dishes in serum free DMEM were used to assay α-synuclein localization. All cell cultures were fixed in 4% paraformaldehyde in. After a quick wash in PBS, 4% PFA in PBS was added for 20 minutes. Following another wash in PBS, fixed cells were stored at 4°C. Fixed cells were permeabilized with PBS-0.1% Triton at room temperature by washing 3 x 10 minutes. Non-specific sites were saturated with PBS-0.1% Triton + 3% BSA + 1% Normal Goat Serum for 90 minutes at room temperature; cell line non-specific sites were saturated with PBS-0.1% Triton + 1% BSA + 5% Normal Goat Serum for 90 minutes at room temperature. Cells were then incubated overnight at 4 ° C with primary antibodies diluted in PBS-Triton + 1% BSA. Cells were washed 3 x 5 minutes in PBS before the incubation with fluorescent secondary antibodies diluted in PBS-Triton + 1% BSA for 90 minutes at room temperature. To remove non-specific signals, 3 x 5 minutes additional washes in PBS were performed. Nuclei were stained by incubating cells with Hoechst solution (2μg/ml), a dye that binds to chromatin. After a quick wash in PBS, Pro Long Gold Antifade Reagents (Life Technologies) was used to mount fixed and stained cells. Stained cells were photographed with a fluorescence microscope (Eclipse Hoechst staining TE 2000-S microscope; Nikon, Tokyo, Japan) equipped with an AxioCam MRm (Zeiss, Oberkochen, Germany) digital camera.

Primary antibody	Dilution	Secondary antibody	Dilution
PNP (Santa Cruz)	1:2000	Anti-rabbit FITC (Sigma-Aldrich)	1:500
α-synuclein (Santa Cruz)	1:500	Anti-rabbit-fitc (Sigma-Aldrich)	1:500
LAMP1 (Santa Cruz)	1:300	Anti-mouse-tritc (Sigma-Aldrich)	1:500
MAP LC3 β2 (Santa Cruz)	1:500	Anti-mouse-tritc (Sigma-Aldrich)	1:500

3.10 NITRIC OXIDE DETECTION ASSAY

Greiss assay (Ricart-Jané et al. 2002) allows the identification of chemical products from nitric oxide in the conditioned medium.

Pathological conditions such as treatment with different concentrations of pro-inflammatory agents and overexpression of wild type or mutant SOD1 and wild type or mutant α-synuclein in glial cells induce activation of glial cells and consequently an increase in the activity of inducible nitric oxide synthase (iNOS). Greiss assay was used to quantify the release of nitric oxide in the culture medium.

A nitrate standard curve was performed with $NaNO_2$ at known concentrations from 50 μM to 0.39 μM in order to quantify nitrite concentration in the medium. 5 mM sulfanilamide (Sigma-Aldrich) was added to culture media and the standard curve. Sulfanilamide reacts with nitrite under acid conditions to form a diazonium cation, which subsequently couples with N-1-naphthyl-ethylenediamine dihydrochloride (NEDA 40mM; Sigma-Aldrich) to produce a colored azo dye. After 15 minutes of incubation at room temperature in the dark, absorbance was read at 540 nm in a Multiplate Spectophotometric Reader (Bio-Rad Laboratories).

3.11 HOECHST STAINING AND NUCLEI COUNTING

Hoechst 33258 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460-490 nm. Hoechst preferentially binds to adenine-thymine (A-T) DNA regions, into the minor groove, and exhibits distinct fluorescence emission spectra that are dependent on dye:base pair ratios.

To determine the effect of glutamate on CGNs in presence or absence of microglia or astrocytes overexpressing wild-type or mutant SOD-1, co-culture nuclei were stained with Hoechst 33258 (2μg/ml; Sigma-Aldrich) for 5

minutes at room temperature. Hoechst stain was also used to determine the viability of N9 and IMA2.1 cells overexpressing wild-type and mutant α-synuclein.

Stained cultures were photographed with a fluorescence microscope (Eclipse Hoechst staining TE 2000-S microscope; Nikon, Tokyo, Japan) equipped with an AxioCam MRm (Zeiss, Oberkochen, Germany) digital camera and nuclei counting was performed on 5 random fields from each Petri dish by using ImageJ NIH software. Cell survival was evaluated by counting normal and condensed nuclei (size between 4.2 and 4.8 μm). A 5-minute incubation is enough to stain CGNs nuclei. At this condition, Hoechst hardly stains microglia and astrocyte nuclei, which usually require at least 20 minutes of incubation (Polazzi et al., 2015). Microglial nuclei that occasionally stain can be excluded because they are oval (not round as CGN nuclei) and considerably larger. Condensed nuclei were easily distinguished from normal nuclei by their size, their fragmented morphology, and also by their brightness, being more intense in condensed nuclei than in normal ones.

3.12 WESTERN BLOT

Western blot is a biochemical technique used to identify a protein of interest, thanks to the electrophoretic separation of proteins in a polyacrylamide gel, the subsequent protein transfer onto a nitrocellulose membrane, and the use of a specific primary antibody to recognize the protein of interest bound to the nitrocellulose membrane.

Western Blot allowed us to verify the release of proteins in the conditioned medium and to evaluate the expression of proteins in cell lysates. 500 μl of conditioned medium were collected and concentrated using Microcon-YM-3 filters (EMD Millipore Corporation, Billerica, MA, USA) and resuspended in 15 μl of Loading buffer 4 X (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, DTT 1:5 of the total volume), while parallel cell samples were collected in Lysis Buffer (1 % SDS, 50 mM Tris pH 7.4, 1 mM EDTA, 10 μl/ml protease inhibitor cocktail and 10 μl/ml phosphatase inhibitor cocktail), sonicated and protein content quantified

by the Lowry assay (Lowry et al. 1951). A BSA (Sigma-Aldrich 1,5mg/ml) standard curve was performed at known protein concentrations in order to quantify the total amount of protein in each sample. Solution 1 consisting of 98% of solution A (2% Na_2CO_3 in NaOH 0.1M), 1% of solution B ($CuSO_4 \cdot 5H_2O$ 0.5%) and 1% of solution C (1% Na-K tartrate) was added to each sample and the standard curve. Subsequently solution 2 (50% Folin and Ciocalteu's phenol reagent and 50% distilled water Bi) was added and after 30 minutes absorbance was read in a spectrophotometer at 700 nm in order to determine total protein concentration.

Concentrated conditioned media and cell samples resuspended in Loading Buffer 4X (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; Bromophenol Blue of 20 mg) plus DTT 1:5 of the total volume) were boiled and loaded into a 10% or 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; Bio-Rad). Acrylamide and protein markers were supplied by Bio-Rad, while the other reagents were from Sigma-Aldrich. Gel electrophoresis was run at constant 120 V in an electrophoretic Mini-Protean II cell (Bio-Rad) filled with 1X running buffer (H_2O bd; 25 mM Tris base; 192 mM Glycine; 0.1% SDS). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (GE Healthcare). Transfer from the cathode to the anode was carried out at constant 400 mA for 2 hours in the Mini Trans-Blot Cell system (Bio-Rad), with 1X transfer buffer (50 mM Tris, 200mm glycine, plus 20% methanol.

Membranes were blocked for 1h with a blocking solution made of 4% nonfat dried milk (Bio-Rad)/0.1% Tween-20 in PBS (Sigma-Aldrich), pH 7.4 and incubated over night at 4 °C with primary antibodies in PBS-0.1% Tween 20. Membranes were washed 3 x 10 minutes with blocking solution and incubated with specific secondary antibodies conjugated to horseradish peroxidase for 90 minutes at room temperature in PBS-0.1% Tween 20, pH 7.4. Membranes were then washed 3 x 10 minutes with PBS-0.1% Tween 20, pH 7.4 and 5 minutes with PBS. Labeled proteins were visualized by using the Clarity^{TM} Western ECL Substrate (Bio-Rad) and detected using a ChemiDoc™ MP imaging system and Image Lab software (Bio-Rad).

Primary antibody	Dilution	Secondary antibody	Dilution
SOD1 (Santa Cruz)	1:1000	Anti-rabbit (Santa Cruz)	1:2000
LAMP1 (Santa Cruz)	1:500	Anti-mouse (Santa Cruz)	1:2000
MRC1 (Santa Cruz)	1:1000	Anti-goat (Santa Cruz)	1:4000
TREM2 (Santa Cruz)	1:1000	Anti-rabbit (Santa Cruz)	1:2000
iNOS (Santa Cruz)	1:1000	Anti-rabbit (Santa Cruz)	1:2000
MAP LC3 β2 (Santa Cruz)	1:1000	Anti-mouse (Santa Cruz)	1:2000
GAPDH (Santa Cruz)	1:5000	Anti-mouse (Santa Cruz)	1:2000
B-Actin (Sigma-Aldrich)	1:1000	Anti-rabbit (Santa Cruz)	1:2000
PNP (Santa Cruz)	1:2000	Anti-rabbit (Santa Cruz)	1:2000
α-synuclein (Santa Cruz)	1:1000	Anti-rabbit (Santa Cruz)	1:1000
Phospho-mTOR	1:1000	Anti-rabbit (Santa Cruz)	1:2000
mTOR	1:1000	Anti-mouse (Santa Cruz)	1:2000

3.13 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High Performance Liquid Chromatography (HPLC) is a technique that allows the separation of two or more compounds (analytes) in solution. The separation process is based on analyte affinity to the stationary phase located within the chromatographic column, and the mobile phase that flows through the column. Because the stationary phases packaged in columns consist of fine particle (3-5 μm), it is necessary to flow the mobile phase at an elevated pressure (50-150 atm) that allows flows of a few ml/min. Solutes are separated in the column and then analyzed by a detector able to send signals to a computer that quantifies data with a chromatogram. The signal related to each analyte is identified by a peak and quantified by its height or by the area under the peak. In order to associate each peak to any component of the sample and quantify it, a standard solution of known composition and title was provided. HPLC was used to quantify the release of glutamate and serine in conditioned media from transfected microglia and astrocytes exploiting the liquid-liquid partition chromatography in reverse phase. A Beckman Coulter Ultrasphere ODS column packed with the stationary phase formed by 5 μm diameter particles was used. 20 μl of each medium were introduced into the flow. The detector (Varian 9070) can reveal glutamate and serine coming out (eluate)

from the column. To measure the amount of glutamate and serine, standard solutions of serine and glutamate at concentrations between 0.625 µM and 20 µM were used.

3.14 QUANTITATIVE REAL TIME PCR

Real Time PCR (qRT-PCR) is a technique that allows DNA amplification and quantification by measuring DNA amplification during the exponential phase of the polymerase chain reaction.

In order to detect PNP and α-synuclein mRNA expression in microglia, astrocytes and cerebellar granule neurons, real-time PCR was performed on extracted total RNA. For total RNA extraction, 2×10^6 microglial cells, 0.5×10^6 astrocytes and 2.4×10^6 CGNs were lysed in 1 ml of Tri-reagent (Sigma-Aldrich), according to the manufacturer's protocol. The first step consisted in treatment with chloroform and centrifugation to separate 3 phases: the aqueous phase containing RNA, the interphase with DNA and the bottom organic phase containing proteins. Isopropanol was added to the aqueous phase which was subsequently centrifuged and the RNA pellet was washed in 75% ethanol and allowed to dryRNA pellets were resuspended in DEPC diethyl pyrocarbonate)-treated deionized water (Sigma-Aldrich). RNA levels were quantified using a NanoDrop UV spectrophotometer (Thermo scientific, NanoDrop Products, Wilmington, DE, USA) and stored at −80°C until used for cDNA synthesis. . In order to remove any DNA contamination, treatment with DNase I (DNase I, RNase-free, Thermo-Fisher Scientific) was used. For each sample, 1 µg of DNase-treated RNA was retro-transcribed using the Superscript III First-Strand Synthesis SuperMix for qRT-PCR kit (Life Technologies) following the manufacturer's protocol. cDNAs were stored at −20°C until used for qRT-PCR.

Rat PNP, α-synuclein and β-actin qRT-PCR primers were designed as follows:

Rat PNP

Forward: 5' ATCCGTGACCACATCAACCT 3'

Reverse: 5' TTGAAAGCCTTCTGCCTCAT 3'

Rat α-synuclein

Forward: AGACCAAAGAACAAGTGACAA

Reverse: TCTTCACCCTTGCCCATCTG

Rat β-Actin

Forward: 5′ AGCAGATGTGGATCAGCAAG 3′ Tm

Reverse: 5′ AACAGTCCGCCTAGAAGCAT 3′ Tm

cDNA samples were diluted to a final concentration of 20 ng/µl. A 2X master mix of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Segrate, Italy) was mixed with the adequate primers (final concentration 0.2 µmol/l), cDNA (40 ng per replicate) and deionized water. qRT-pCR was performed using an iCycler IQ (Bio-Rad) thermal cycler and real-time PCR measurements were carried out in duplicate with three biological replicates. mRNA relative expression levels were normalized using β-actin as the internal control.

Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as difference between the target genes and the expression of the endogenous gene β-Actin. $\Delta\Delta^{Ct}$ values were calculated as differences to control sample; $\Delta\Delta^{Ct}$ values were expressed as percentage of values measured for controls.

3.15 STATISTICAL ANALISYS

All quantitative data are expressed as means ± SE from independent experiments. Statistical significance between different treatments was calculated with GRAPHPAD PRISM 6 (La Jolla, California, USA) by using one-way analysis of variance (ANOVA) followed by post-hoc comparison through Bonferroni's test. A value of $p < 0.05$ was considered statistically significant.

4. RESULTS

4.1 PNP RELEASE BY GLIAL CELLS

4.1.1 PNP EXPRESSION AND RELEASE BY RAT PRIMARY CULTURES OF MICROGLIA, ASTROCYTES AND DIFFERENTIATED CGNS

Purine Nucleoside Phosphorylase (PNP) localization in the brain seems to be restricted to glial cells, while its expression in neurons is still debated (Castellano et al., 1990). Because of the crucial role of microglia in the development of the central nervous system by controlling purine metabolism, we decided, as a first step, to check the level of PNP expression in primary cultures of glial cells and cerebellar granule neurons. The expression of PNP was evaluated by different methods. By using immunofluoresce, we observed that microglia and astrocytes expressed high levels of PNP, with marked staining in all examined cells, mainly located in the nucleus and cytoplasm, while differentiated CGNs showed a robust nuclear labeling, with almost no staining on neurites (Fig. 1A). The difference in PNP expression between glial cells and neurons was confirmed by real-time PCR, showing the highest levels of PNP mRNA in microglial cells and the lowest in differentiated CGNs (Figure 1B). Western blot analysis (Fig. 1C) confirmed the presence of PNP protein in all neural cells, even though it was more significantly expressed in glial cells than in CGNs. In order to study whether PNP was released by the different types of brain cells in culture, we performed western blot analysis to evaluate the amount of PNP protein in media conditioned for different times by primary microglia, astrocytes or neurons. As shown in figure 1D, western blot analysis revealed that PNP was constitutively released and accumulated in the culture medium (conditioned medium, CM) by microglia (MCM) or astrocytes (ACM) and, to a lesser extent, in medium conditioned by CGNs (CGN-CM) for 6 and 24 hours.
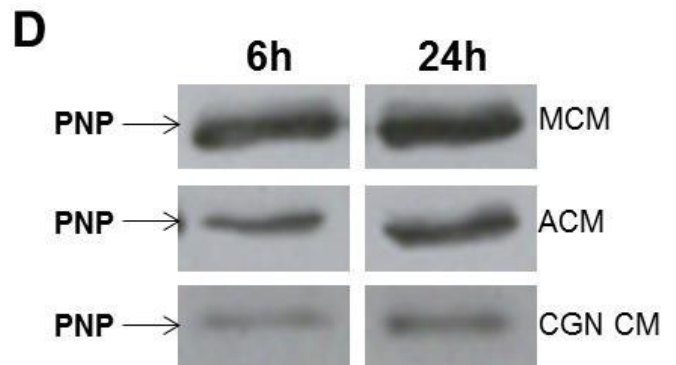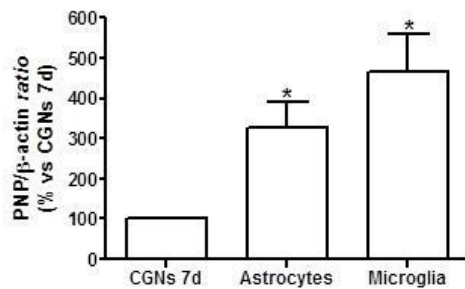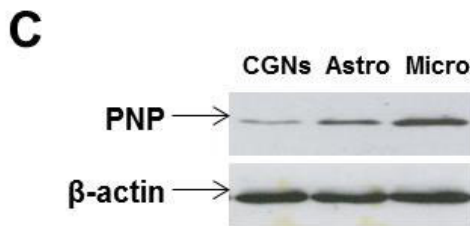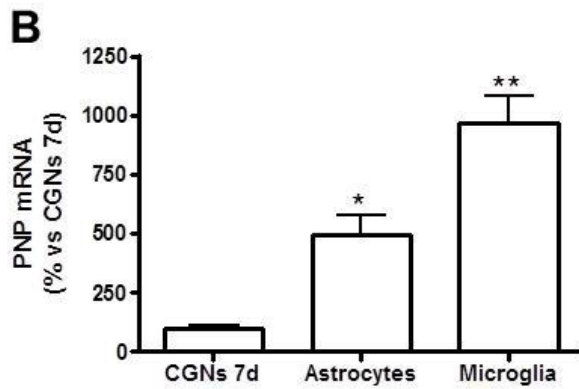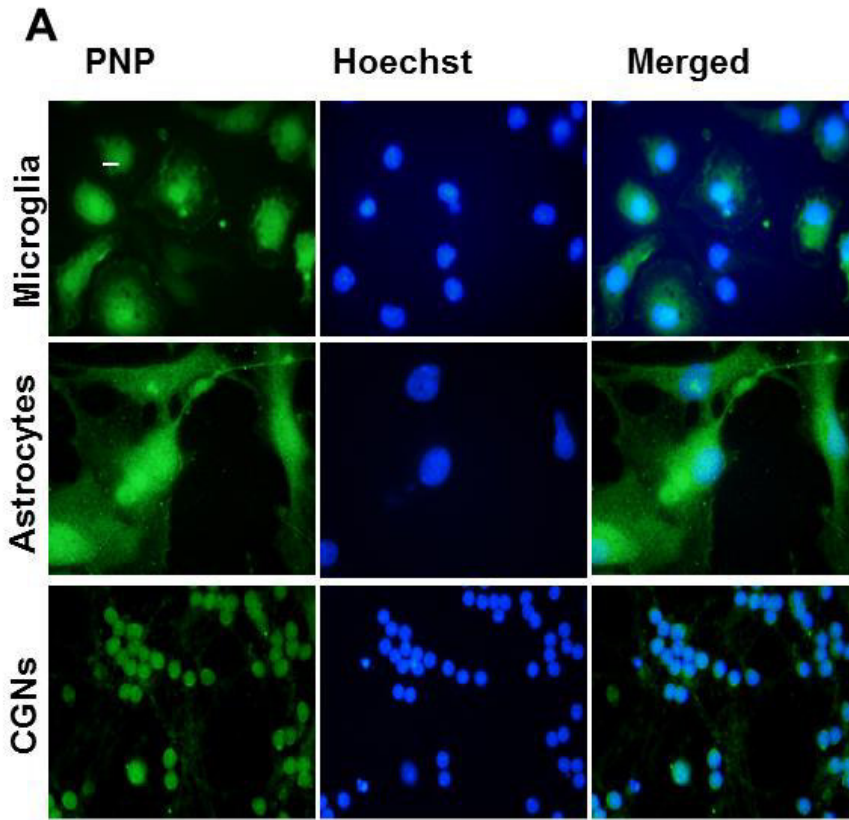
Fig. 1. PNP expression in rat primary microglia, astrocyte and cerebellar granule neuron (CGNs) cultures.

Immunofluorescence analysis (1A) of PNP expression (green) in rat primary cultures with nuclear Hoechst chromatin staining (blue) (Scale bar: 15 µm). PNP mRNA expression in rat primary microglia, astrocyte and differentiated cerebellar granule neuron cultures was analyzed by qRT-PCR (1B). Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as the difference between the target gene and the expression of the endogenous gene β actin. $\Delta\Delta^{Ct}$ values are the mean ± S.E. of 3 different samples and are expressed as percentage versus differentiated CGN values. Western Blotting analysis of PNP expression (1C) and PNP release (1D) from rat primary microglia, astrocyte and differentiated cerebellar granule neuron cultures. Cells were plated in serum free medium at a density of 1.5×10^6 cells/1ml for microglia, 0.5×10^6 cells/1ml for astrocytes 2.4×10^6 cells/2ml for differentiated cerebellar granule neuron cultures per 35mm Ø Petri dish. To assay protein release, 500µl of serum free conditioned medium collected at 6 or 24 hours after culture were concentrated.

4.1.2 INFLAMMATORY STIMULI DO NOT MODIFY CONSTITUTIVE PNP SECRETION FROM CULTURED MICROGLIA, ASTROCYTES AND DIFFERENTIATED CGNS

One of the first stimuli described in literature able to activate microglia was the bacterial endotoxin lipopolysaccharide (LPS) (Hetier et al., 1988). *In vitro* activation of microglia induced by LPS mimics the glial activation that occurs *in vivo* due to inflammatory stimuli, but also the chronic microglial activation due to neurodegenerative diseases (Hetier et al., 1988). Activation increases the production and release of pro-inflammatory cytokines, as well as chemokines (Kong et al., 1997; Lieberman et al., 1989), and up-regulates nitric

oxide synthase (NOS) (Pahan et al., 1997; Boje and Arora, 1992) mediated by the TLR4 receptor (Takeda et al., 2003).

In several neuropathological situations, interferon-γ (IFN-γ) is produced by activated T-lymphocytes and natural killer cells, modulates immunological responses and stimulates the production of reactive nitrogen and oxygen species and cytokines/chemokines in microglia (Schroder et al., 2004).

Because glial cells play a crucial role in brain pathophysiology, mainly as consequence to their activation, we assessed whether PNP release could be influenced by typical inflammatory stimuli, such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Primary microglia, astrocyte and differentiated CGNs cultures were exposed for 24 hours to increasing concentrations of LPS and IFN-γ; CMs were then collected and analyzed by western blot analysis, in parallel with corresponding cell lysates. None of the two stimuli affected the amount of PNP neither in cells nor in the medium conditioned by microglia (Fig. 2A-B), astrocytes (Fig. 2C-D) or CGNs (Fig. 2E-F).
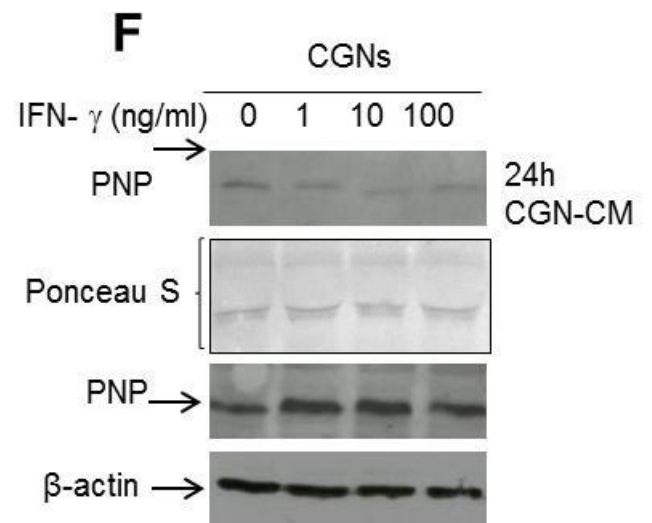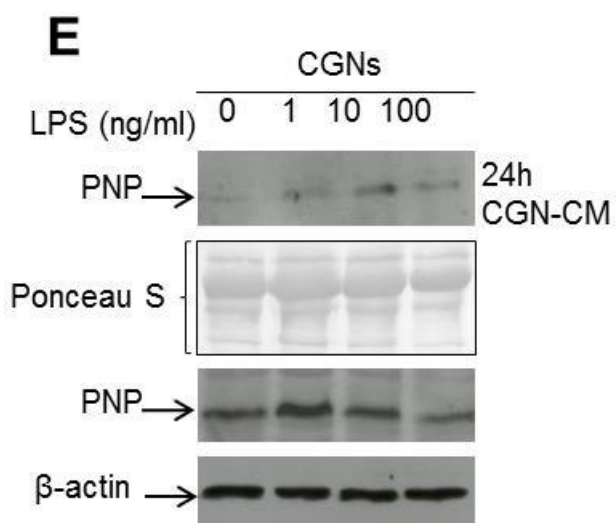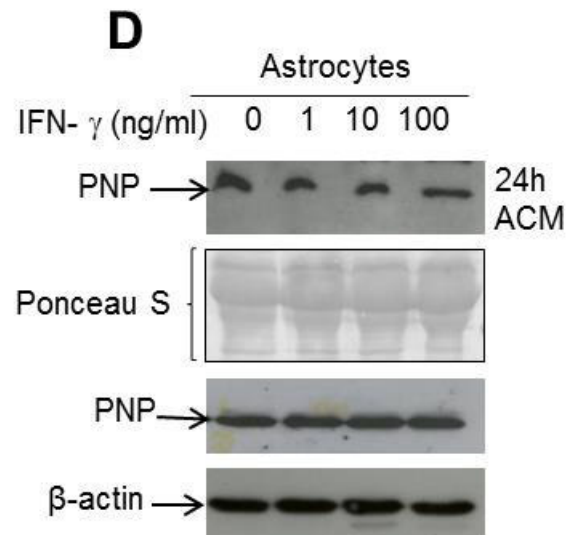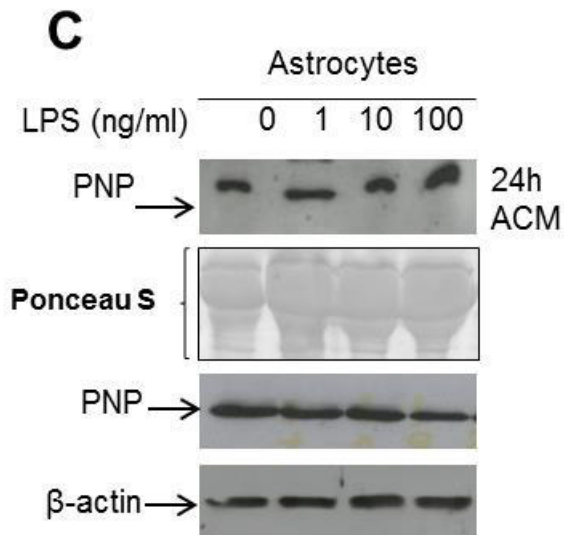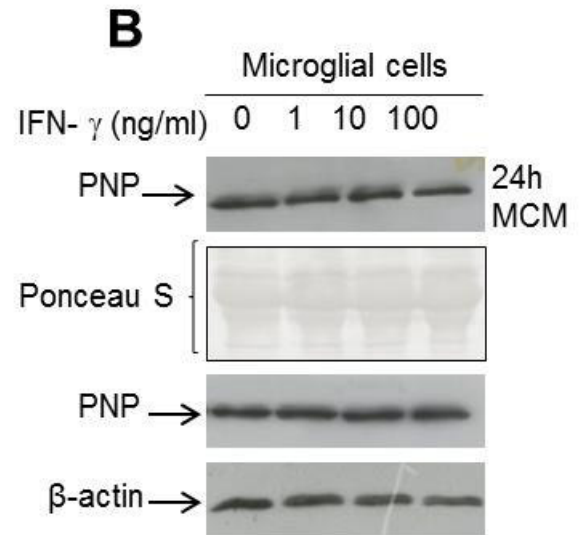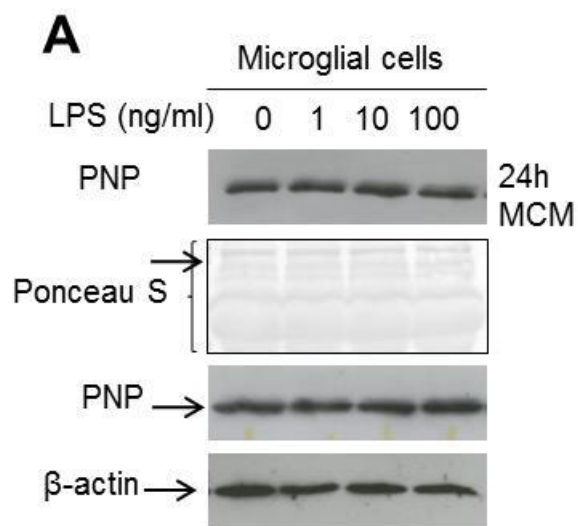
Fig 2. Effect of activation through lypopolysaccharide (LPS) and interferon-γ (IFN-γ) on PNP release by microglia, astrocytes and cerebellar neurons.

Representative western blot analysis of PNP protein released in conditioned media (upper lanes), PNP protein expression (middle lanes) and β-actin expression as loading control (lower lanes) in whole cell lysates from rat primary microglial (2A- 2B), astrocyte (2C- 2D) and cerebellar neuron (2E-2F) cell cultures stimulated for 24 hours with increasing LPS concentrations of LPS [1; 10; 100 ng/ml] (2A- 2C-2E) or IFN-γ [1; 10; 100 ng/ml] (2B- 2D- 2F). Equal volumes (500 μl) of 24h-conditioned medium from microglia (MCM), astrocytes (ACM) and differentiated cerebellar granule neurons (NCM) were concentrated and loaded in parallel with 20μg of total protein lysate from the same cell cultures. Equal loading was confirmed by staining with Red Ponceaus S performed on membranes before immunodetection.

4.1.3 ATP ENHANCES CONSTITUTIVE PNP SECRETION FROM CULTURED MICROGLIA AND ASTROCYTES

It is known that ATP is abundantly released by all neuronal cells and represents one of the most important neuro/glio-transmitters. In neuropathology, ATP is released by damaged cells and acts both as a cytotoxic factor and a pro-inflammatory mediator, being a universal "danger" signal (Franke, et al., 2012).ATP-mediated activation through the activation of P2X7 receptors appears not to be chronic, but less aggressive than the activation mediated by LPS and mainly leading to an autophagic apoptotic condition (Takato et al., 2009). Thus we wondered whether it could affect/modulate PNP secretion in the examined cells. Exposure of microglial cells (Fig.3A) and astrocytes (Fig.3B) to ATP enhanced PNP release in the extracellular medium after 1h.
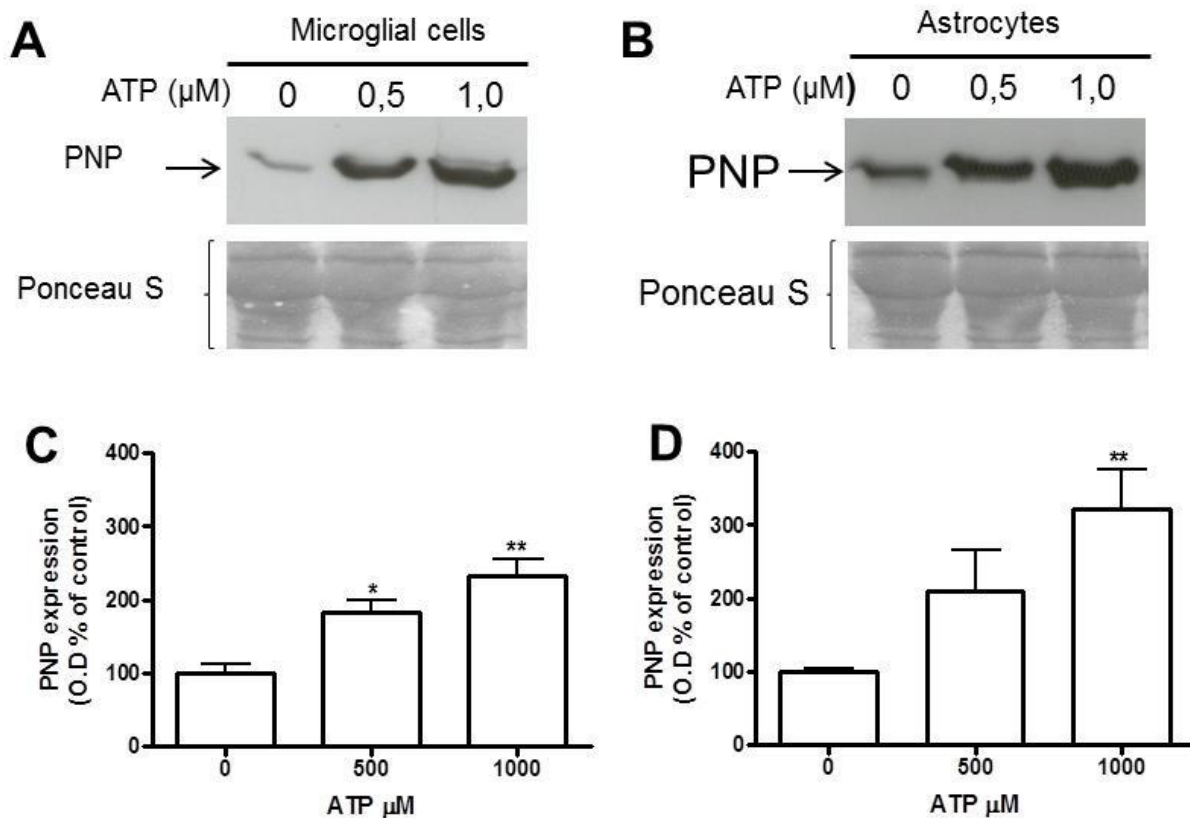
Fig.3. Effect of ATP on PNP extracellular release in primary cultures of rat microglia and astrocytes.

Western Blot analysis (3A-3B) and relative densitometries (3C-3D) of PNP release in media conditioned for 1 hour by primary rat microglia and astrocyte cells treated with increasing ATP concentrations [0, 0,5 or 1 mM]. 500 μl of conditioned medium were concentrated and loaded to assay PNP release. Data are expressed as percentage of controls and are the mean ± s.e. from three independent experiments. *p < 0.05; **p < 0.01 compared to controls. Equal loading was confirmed by staining with Red Ponceaus S performed on transfered membranes.

4.1.4 PNP RELEASE IS MEDIATED VIA THE LYSOSOMAL SECRETORY PATHWAY

Since ATP is known to induce protein release through the lysosomal secretory pathway (Dubyak, 2012; Takenouchi, 2009), we verified whether this

pathway could be involved also in PNP release. By immunofluorescence, we observed in astrocyte (Fig. 4A) and microglia (Fig. 4B) cultures that PNP staining co-localized with LAMP-1, a well-known lysosomal protein. Interestingly, the exposure of brain cell cultures to ATP determined a scattered distribution of both PNP and LAMP-1 signals, possibly in agreement with activation of lysosomal release.

Since glial secretion through extracellular vesicles (EVs) has acquired increased importance in physiological glial-neuron communication, we assessed whether PNP protein could be released by glial cells this way. EVs were isolated from MCM and ACM collected after 2 hours of 500 µM ATP treatment or control exposure of cultured cells. Western blot analysis revealed the presence of PNP in both the soluble (SF) and the pellet fraction (PF, containing EV) from both MCM and ACM, mainly in conditioned media from ATP-stimulated cells (Fig. 4C-D), being the pellet fraction also positive for LAMP-1. These data demonstrate that microglia and astrocytes release PNP through both a classical and an EVs-mediated release, the latter occurring through the lysosomal secretory pathway upon exposure to ATP.
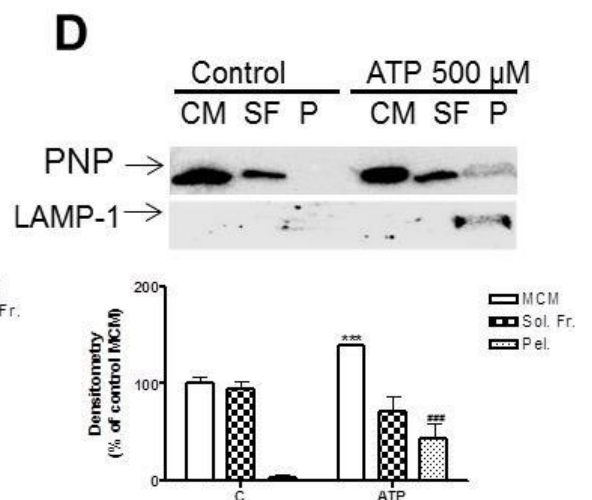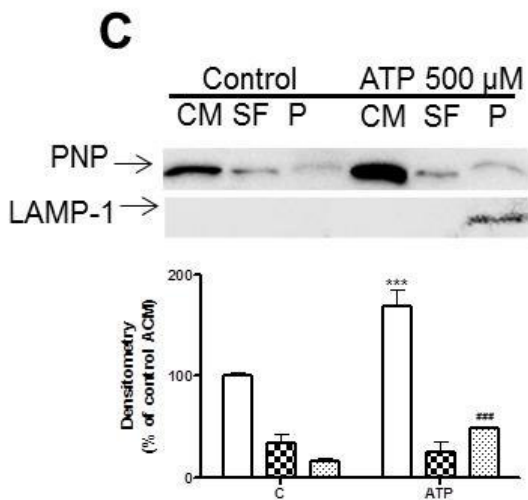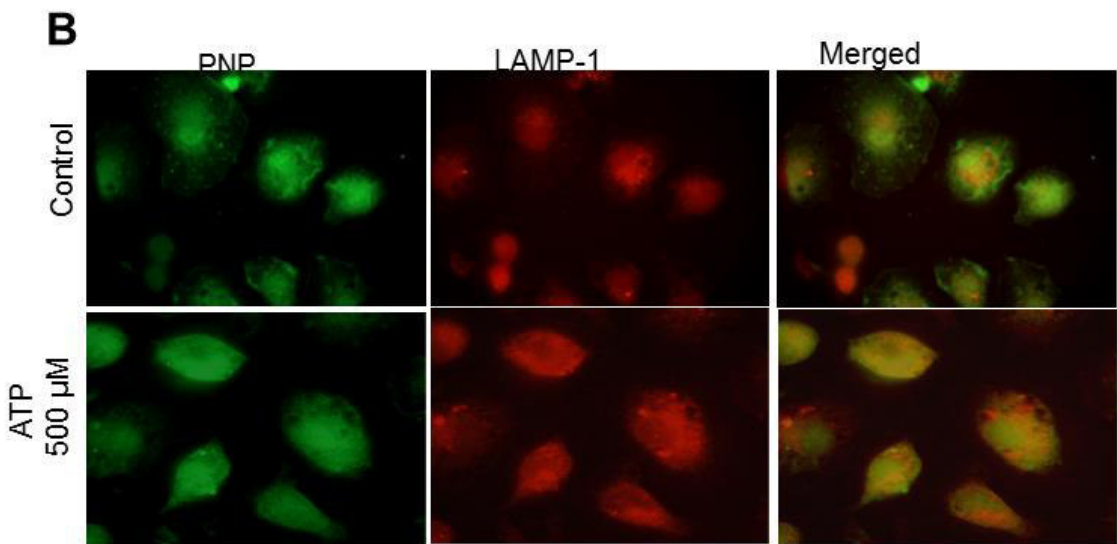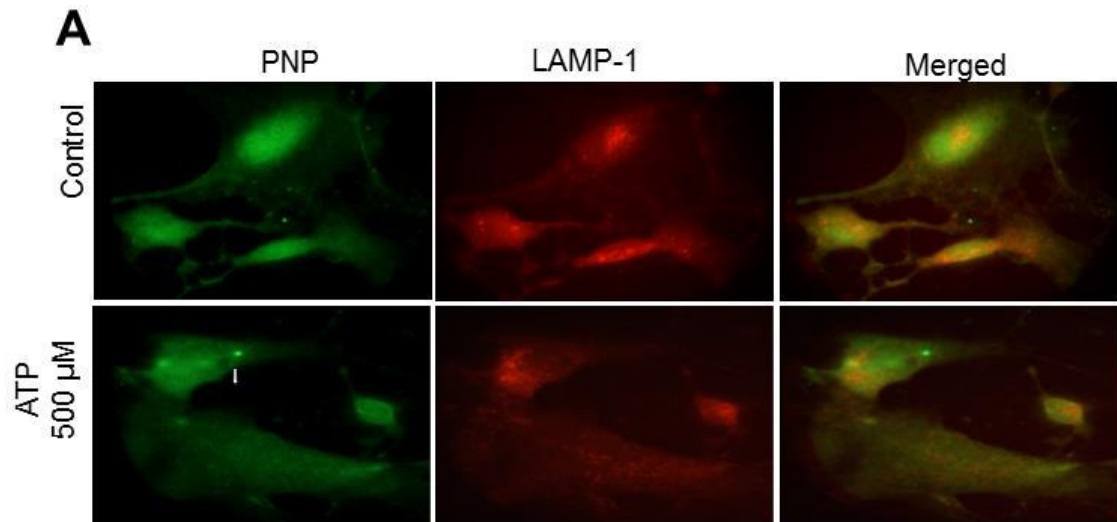
Fig. 4. Co-localization of PNP with the lysosomal protein LAMP-1 in astrocytes and microglia and consequent PNP release via extracellular vesicles (EVs).

Immunofluorescence analysis of PNP (green) and LAMP-1 (red, lysosomal marker) expression in microglia (4A) and astrocyte (4B) cell cultures in basal conditions (control) and after stimulation with 500 μM ATP for 1 hour (lower lane) (Scale bar: 15 μm). PNP and LAMP-1 protein expression evaluated by Western blot analysis and relative densitometry analysis (D, F) performed on aliquots of 2 hour-conditioned media (500μl) collected from rat microglial (4C) and astrocyte (4D) cultures and on samples from the soluble (SF) or pellet fractions (PF) isolated from the same MCM and ACM treated or not with ATP (500 μM). Images are representative of three independent experiments. Each bar represents the mean ± S.E. PNP protein expression as percentage *versus* control conditioned medium *** p < 0.001; PNP protein expression as percentage *versus* ATP conditioned medium ### p < 0.001. One way ANOVA followed by Bonferroni's multiple comparison test.

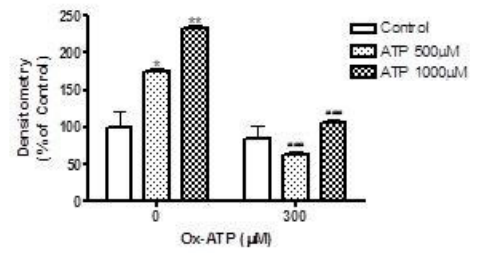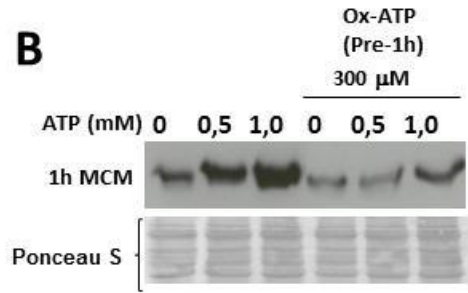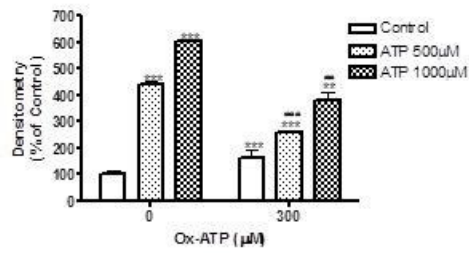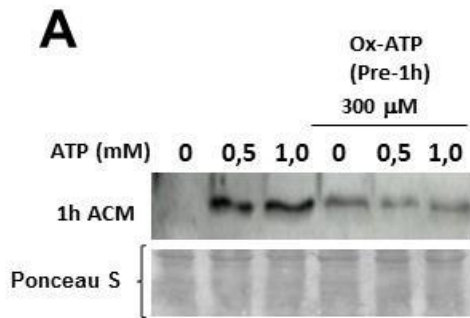## 4.1.5 INVOLVEMENT OF P2X7 RECEPTORS IN PNP RELEASE

Since ATP was effective on PNP release in both types of glial cells, we compared the effect of ATP with 3'-O-benzoyl-benzoyl-ATP (Bz-ATP), a stable form of ATP, used in a range of selective concentrations able activate P2X7 receptors [125, 250 μM] (Young et al., 2007). At the same time, two non-selective P2 receptor antagonists were used: oxidized ATP (ox-ATP) and suramin. Oxidized ATP (ox-ATP) is a selective inhibitor of the P2X7 receptors (Lang et al., 2010). Suramin is a non-selective antagonist of P2 receptors and, similarly to ox ATP, it could inhibit immune response but, at the same time, it also has anti-inflammatory proprieties. By analyzing medium conditioned by microglia (Fig. 5B-D-F-H) or astrocytes (Fig. 5A-C-E-G), we observed that Bz-ATP treatment in microglia (Fig. 5D-H) and astrocytes (Fig. 5C-G) stimulated PNP release by both cell types in a dose-dependent way. This effect

was even greater than the one caused by cell exposure to 0.5 or 1.0 mM ATP. As expected, cell pre-treatment for 1h with Ox-ATP, a non-competitive antagonist of P2X7 receptor (Michel et al., 2000; Hibell et al., 2001; Bartlett et al., 2014), completely blocked the effect of Bz-ATP on PNP release (Fig. 5C-D) and significantly reduced the effect of ATP (Fig. 5A-B). Cell pre-treatment for 1 hour with suramin, a non-selective antagonist of P2 receptor, did not inhibit PNP release mediated by Bz-ATP (Fig. 5G-H); however, suramin pre-treatment seemed to promote a non-statistically significant increase in ATP-mediated PNP release (Fig.5E-F). Together these findings support the specific involvement of P2X7 receptors in the ATP-mediated induction of PNP release.
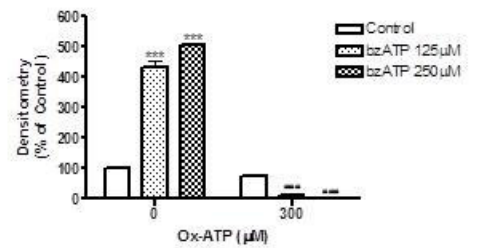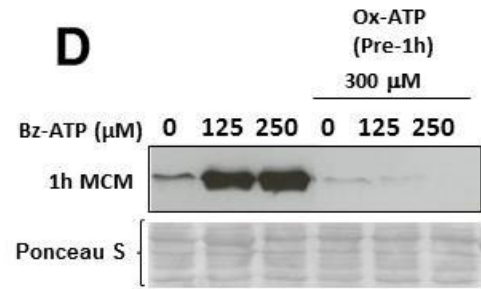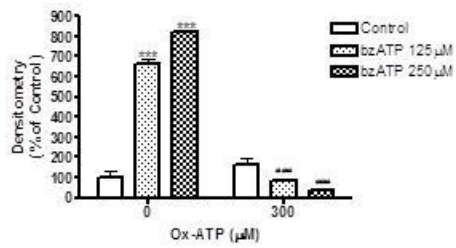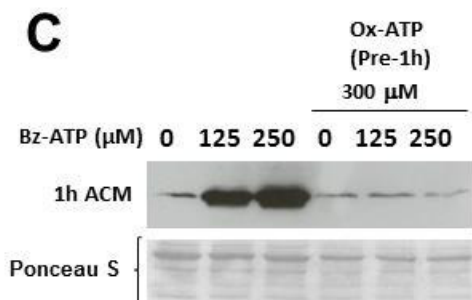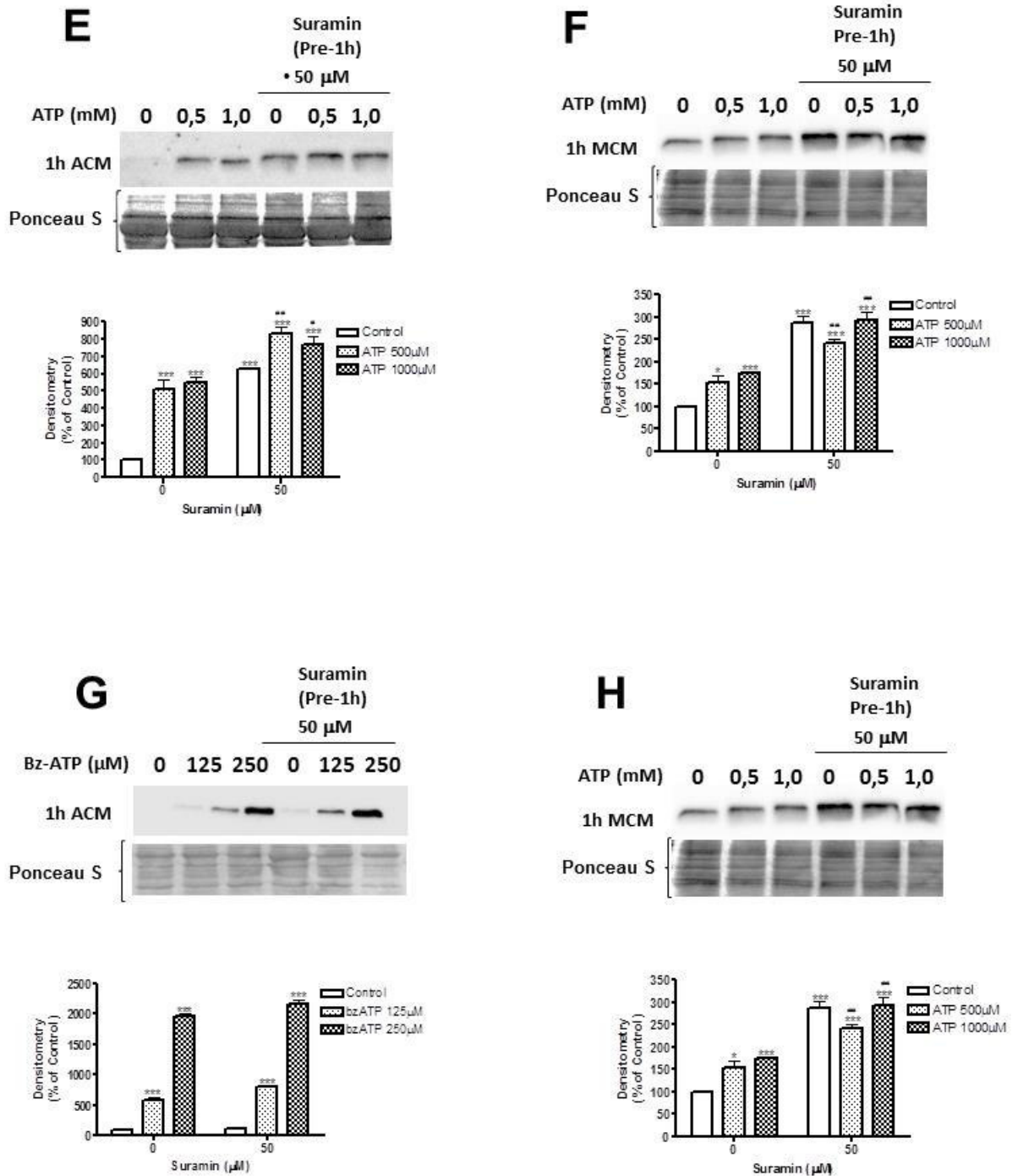
Fig 5. P2X7 receptor involvement in PNP release by primary microglia and astrocytes.

Western Blot analysis and relative densitometry of PNP protein expression in conditioned media (500 µl) for 1 hour by primary rat microglia (B-D-F-H) and astrocyte (A-C-E-G) cells treated with increasing ATP concentrations [0, 0.5 or 1.0 mM] (A-B-E-F) or Bz-ATP [0, 125,250 µM] (C-

D-G-H) and pre-treated with ox-ATP (A-B-C-D) or suramin (E-F-G-H). Equal loading was confirmed by membrane staining with Red Ponceaus S. Data are expressed as percentage *versus* controls and are the mean ± S.E. from three independent experiments. * $p < 0.05$; ** $p < 0.01$ * $p < 0.001$ compared to controls # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ compared to pre-treated controls. One way ANOVA followed by Bonferroni's multiple comparison test.

4.2 SOD1 RELEASE FROM RAT PRIMARY CULTURES OF MICROGLIA AND ASTROCYTES IN PHYSIOPATHOLOGICAL CONDITIONS

4.2.1 MICROGLIAL SOD1 RELEASE THROUGH LYSOSOMES

Previous studies from our lab have shown in rat primary cultures a neuroprotective role of microglial cells against different types of neuronal damage through the release of soluble factors, being SOD1 one of the molecules identified in microglial conditioned medium showing a neuroprotective function (Polazzi et al., 2013). In addition, astrocytes also release SOD1 through exosomes in mouse primary cultures (Basso et al., 2013). Therefore, we decided to check whether also microglial cells release SOD1 through exosomes. Thus, first we performed western blot analysis on media conditioned for 2, 24, and 48 hours by primary cultures of rat microglia (MCM) and astrocytes (ACM). As shown in figure 6, SOD1 is constitutively released by both microglia (Fig. 6A) and astrocytes (Fig. 6B) and accumulates significantly over time in the medium. We then isolated vesicles from 2h-MCM and 2h-ACM in control conditions or after treatment with ATP 500 μM for 2 hours. This stimulation is known to promote vesicular secretion in many immune cells, including macrophages and monocytes, through P2X7 receptors (Dubyak, 2012). Western blot analysis of conditioned media, as well as of the

soluble and pellet fractions, with the latter positive to Lysosome Associated Membrane 1 (LAMP-1), indicates that both microglia (Fig.6C) and astrocytes (Fig. 6D) release SOD1 through a vesicular secretion; this also confirms previously published data of SOD1 secretion through the lysosomal secretory pathway (Polazzi et al., 2013). The presence of vesicular structures with a diameter compatible with exosomes has been confirmed in parallel samples of microglial conditioned medium through atomic force microscopy (Fig. 6E and F).
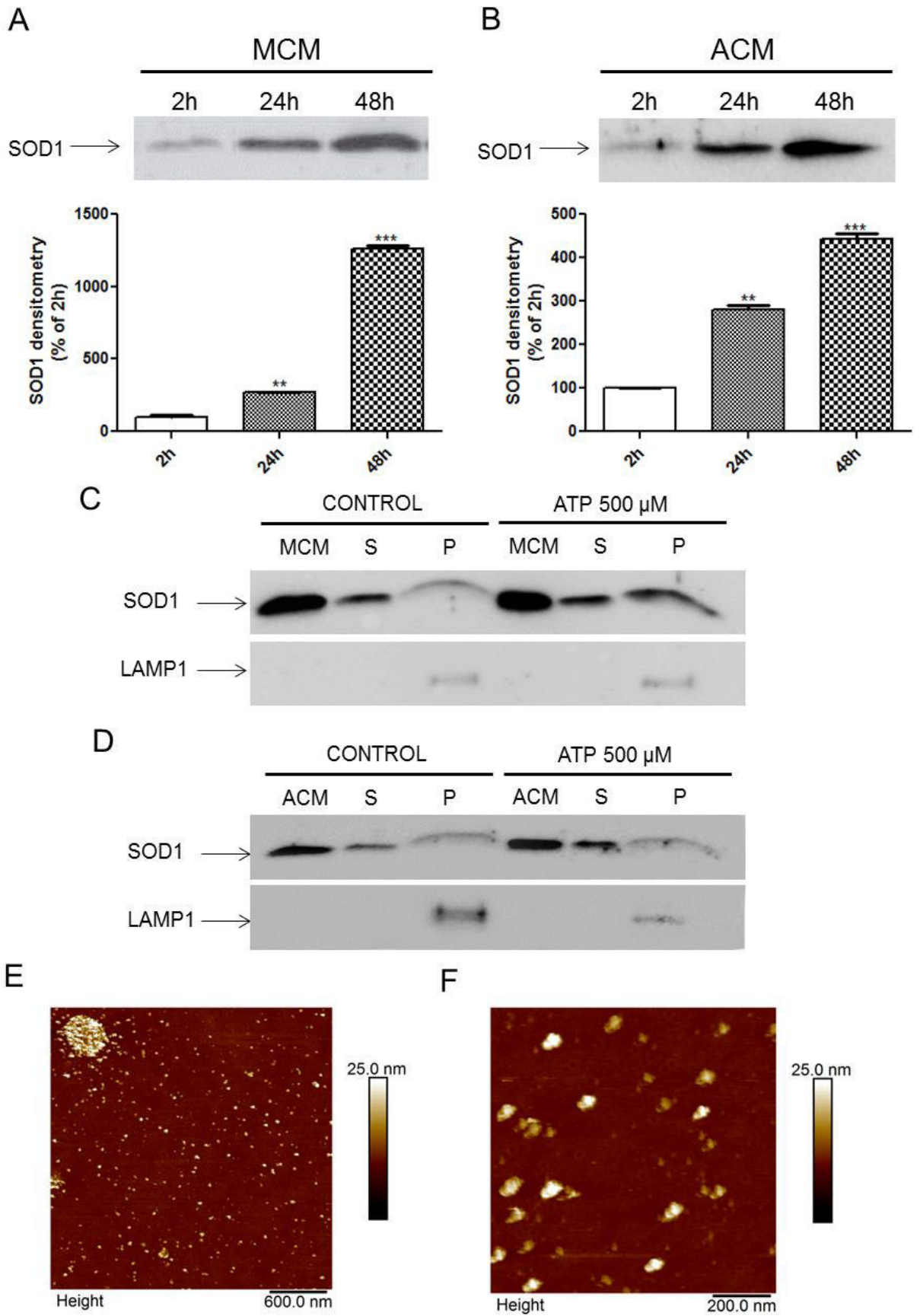
Fig.6. SOD1 release from rat primary cultures of microglia and astrocytes.

Western blot analysis of SOD1 in conditioned medium for 2, 24 or 48h by microglia (A) or astrocytes (B), with relative densitometries and PNP protein as loading control, shows that not only microglia, but also astrocytes release SOD1, which accumulates in conditioned medium. Western blot analysis of media conditioned for 2h by rat primary culture of microglia (C) and astrocytes (D), in control conditions or in presence of ATP 500 μM, indicates that glial cells release SOD1 also through vesicles, since SOD1 is present in the ultracentrifuged pellet, which is also positive for the lysosome-associated membrane protein-1 (LAMP-1). In situ Peak-Force atomic force microscopy images of purified exosomes obtained in buffer at room temperature (E-F). The heights of the features on the substrate are coded according to the attached color table. Exosomes display a relatively narrow size distribution (evident in the large scale image).

4.2.2 INTRACELLULAR MUTANT SOD1 ACCUMMULATION IN PRIMARY CULTURES OF MICROGLIA AND ASTROCYTES

Taking into account both the role of released SOD1 and the non-cell-autonomous hypothesis for ALS neurodegeneration, we decided to study whether the presence of SOD1 expressing mutations linked to familial ALS (G93A and A4V) could alter SOD1 glial release and whether this could be correlated to an alteration of glial phenotype. Therefore, we decided to transiently over-express in rat primary cultures of microglia and astrocytes human wild-type and fALS-linked SOD1 mutants (G93A and A4V) fused with the GFP reporter gene (kind gift from Prof. Poletti, University of Milan, Italy). Western blot analysis performed on the media conditioned for 24 h after transfection with SOD1-WT-EGFP, SOD1-G93A-EGFP, SOD1-A4V-EGFP plasmids shows that both wild-type and mutant SOD1 were expressed and released by microglia (Fig.2A) and astrocytes (Fig.2B). However, the amount of intracellular mutant SOD1 was significantly lower than the amount of wild-type SOD1 and we also observed a significant difference in SOD1 release

between cells that overexpressed the two mutations, being A4V SOD1 released even less than G93A SOD1, accordingly to the severity of disease caused by the mutations.

Interestingly, Western blot analysis performed on the same cells, from which we collected the previously shown conditioned media, showed strong intracellular accumulation of mutant SOD1, with an even more evident accumulation of A4V SOD1, compared to wild-type SOD1 both in microglial cells (Fig.2C) and astrocytes (Fig.2D). The intracellular accumulation of mutant SOD1 was confirmed by immunofluorescence analysis of EGFP SOD1 in parallel cultures of primary rat glial cells. Microglial cells (Fig.2E) and astrocytes (Fig.2F) overexpressing SOD1-G93A-EGFP and specially SOD1-A4V-EGFP demonstrated stronger intracellular accumulation of SOD1, as well as co-localization of SOD1 with the marker of lysosomal vesicles (LAMP-1), than microglial cells overexpressing SOD1-WT-EGFP which was more widespread. Taken together, these data demonstrated for the first time an intracellular accumulation of mutant SOD1 not only in neurons, but also in glial cells.
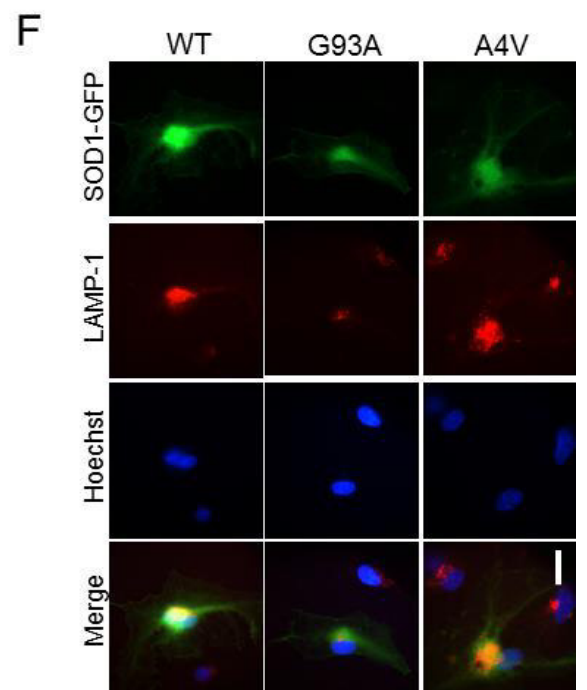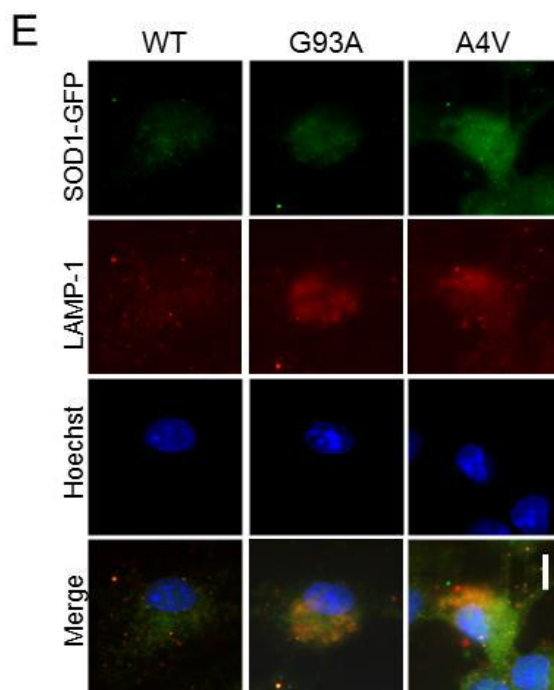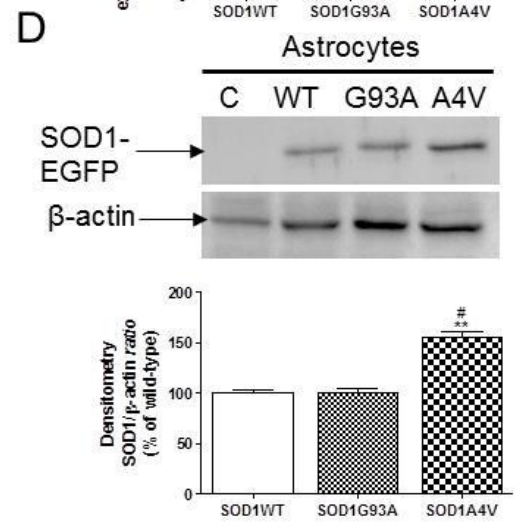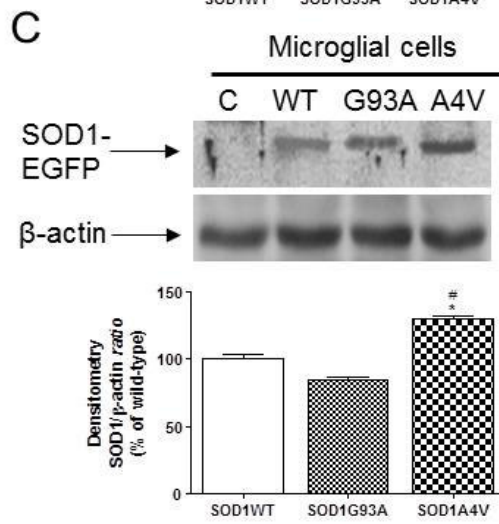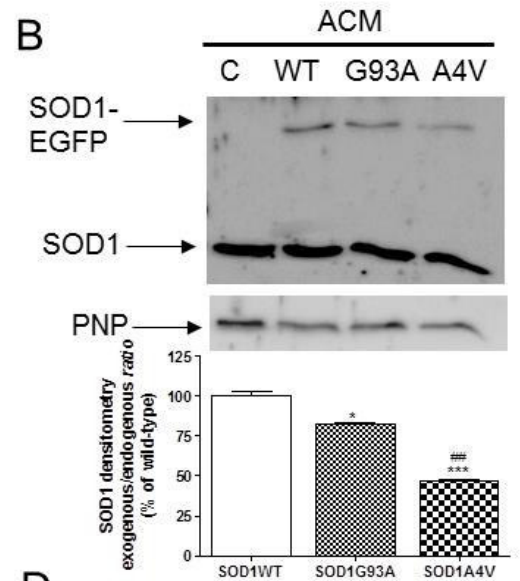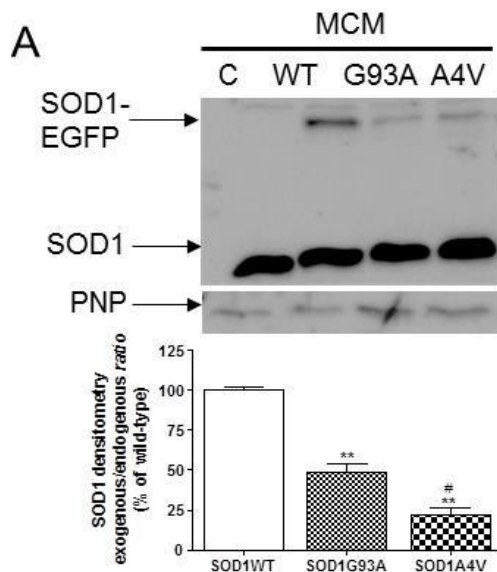
Fig.7. Altered SOD1 release in microglia and astrocytes overexpressing mutant SOD1.

Western blot analysis and relative densitometry of SOD1 released (A, B) and overexpressed (C,D) in 24h conditioned medium by microglia (A) and astrocytes (B) and lysed microglial cells (C) and astrocytes (D) over-expressing wild-type SOD1-EGFP or with mutations linked to familial ALS (G93A and A4V), with the protein PNP as loading control for conditioned media and β-actin for cell lysates. Each bar represents the percentage *versus* SOD1-WT of exogenous/endogenous SOD1 ratio for conditioned media and of SOD1/β-actin ratio in cell lysates. Each bar represents the mean ± S.E. of four samples from different experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT SOD1; # $p < 0.05$, ## $p < 0.01$ compared to G93A SOD1. Bonferroni's post-hoc test following one-way ANOVA. The intracellular accumulation of mutant SOD1 is confirmed by immunofluorescece analysis of SOD1 (green), LAMP-1 (red) and Hoechst (blue) in microglial cells (E) and astrocytes (F). Scale bar = 10 μm.

4.2.3 AUTOPHAGIC PATHWAY ALTERATION IN MICROGLIAL CELLS OVEREXPRESSING MUTANT SOD1

Intracellular accumulation of mutant SOD1 could be due to alteration of intracellular degradation pathways and/or intracellular trafficking, suggesting a dysfunction in the glial autophagic pathway. This hypothesis is also supported by the fact that impairment of the autophagy process seems to contribute to motor neuron death and disease progression in ALS, though it has never been studied in glial cells expressing mutant SOD1 (Lee et al., 2015; Castillo et al., 2013). Here, we studied the expression of LC3 II (microtubule-associated protein 1A/1B-Light Chain 3), which is considered a reliable autophagic marker. During the formation of autophagosomes, the cytosolic protein LC3 I is lipidated to LC3 II and recruited to the autophagosomal membrane (Tanida et al., 2008). Western blot analysis and relative densitometries (Fig. 8A) showed a slight increase in LC3II expression in microglial cells overexpressing

SOD1-G93A-EGFP, but a statistically significant reduction in microglial cells overexpressing SOD1-A4V-EGFP compared to microglial cells overexpressing wild-type-SOD1-EGFP. In parallel conditions, astrocytes seem not to express LC3II. Interestingly, immunofluorescence analysis confirmed the LC3 II reduction and revealed a change in subcellular localization in microglial cells overexpressing SOD1-A4V-EGFP, being LC3II present in the center of microglial cells and not in a vesicular organization, as in control cells (Fig. 8C). In addition, we evaluated another autophagy marker, mTOR (mammalian target of rapamycin), which is a negative regulator of autophagy when phosphorylated on Ser 2448. Through western blot analysis, we observed that the p-mTOR Ser 2448/total m-TOR ratio (Fig.8B) decreases in microglial cells overexpressing SOD1-G93A-EGFP and in a statistically significant way in microglial cells overexpressing the SOD1-A4V-EGFP.
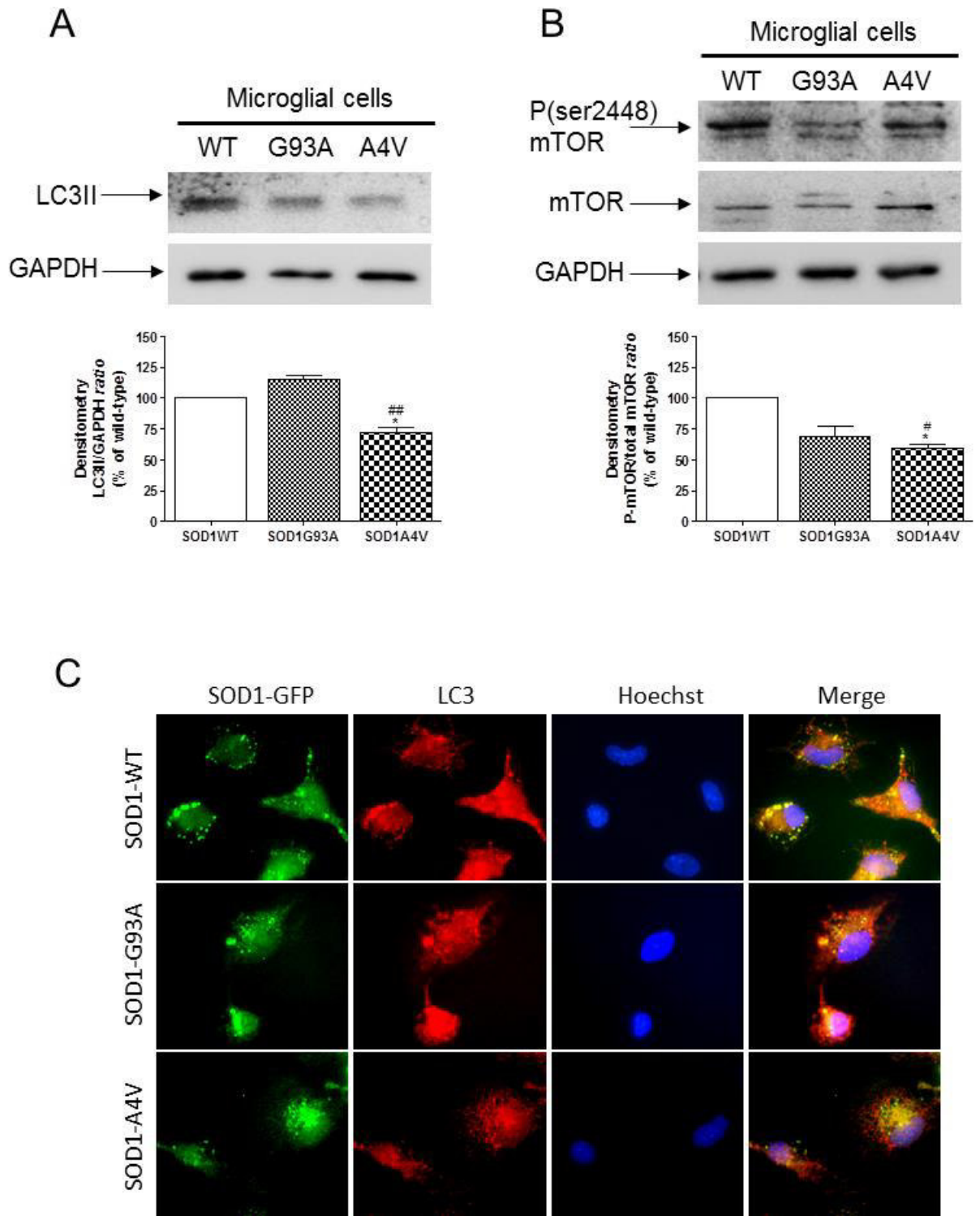
Fig.8. Autophagy markers in microglial cells overexpressing wild-type or mutant SOD1.

Western blot analysis and relative densitometry for the expression of the autophagy marker Light Chain 3 microtubule-associated protein (LC3) (A) and for mTOR phosphorylation on serine 2448 (B). Each bar is the mean ±

S.E. from three experiments of the LC3/GAPDH ratio and of the P(Ser2448)mTOR/total-mTOR ratio expressed as the percentage *versus* WT SOD1. * $p < 0.05$ relative to WT SOD1; # $p < 0.05$, ## $p < 0.01$ relative to G93A SOD1. Bonferroni's post-hoc test following one-way ANOVA. Impairment in the autophagy patway in microglial cells (C) is confirmed by immunofluorescence analysis of SOD1 (green), LC3 (red) and Hoechst (blue) in microglial cells (E) and astrocytes (F). Scale bar=10 μm.

4.2.4 EFFECT OF WILD-TYPE AND MUTANT SOD1 OVEREXPRESSION ON MICROGLIA AND ASTROCYTE ACTIVATION AS WELL AS ON MICROGLIAL PHENOTYPES

Protein intracellular accumulation, observed in many proteinopathies, seems to be responsible for glial cell activation (Ferretti et al., 2012), which is also a typical hallmark of ALS. Given that the increase in intracellular iNOS expression and the consequent release of NO is considered a marker of glial activation (Yuste et al., 2015), we evaluated whether the overexpression of wild-type or mutant SOD1 in glial cells could influence iNOS expression and nitrite production. In microglial cells, mutant SOD1 overexpression slightly increased iNOS expression, as shown by Western Blot analysis (Fig.9A), while in parallel samples nitrite levels in the medium (Fig.9C) were significantly elevated, clearly indicating the activation of microglial cells overexpressing mutant SOD1 compared to wild-type SOD1. On the other hand, overexpression of mutant and wild-type SOD1 in astrocytes did not cause any difference neither in iNOS expression (Fig.9B) nor in nitrite levels (Fig.9D), further underling the different response of the two glial cell types to SOD1 overexpression. Furthermore, considering that microglia activation is often associated with their shift from the M2 neuroprotective to the M1 neurotoxic phenotype even in ALS (Zhao et al., 2013), we evaluated the expression of two cellular markers linked to microglia alternate activation, the Triggering Receptor Expressed on Myeloid Cell 2 (TREM2) and the Mannose Receptor C Type 1 (MRC1), also associated to the microglial phagocytic activity (Lue et

al., 2015; Colton et al., 2006). As shown in figure 9E and 9F, there was no difference in TREM2 and MRC1 expression, suggesting that microglial activation due to mutant SOD1 overexpression leads to the M1 phenotype without losing M2 features and that no differences are evident between the two SOD1 mutations.
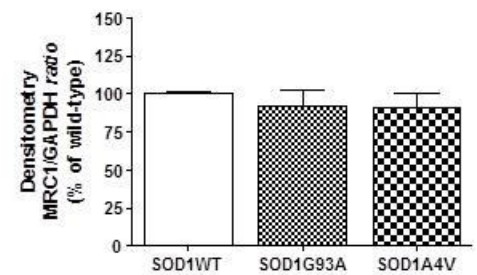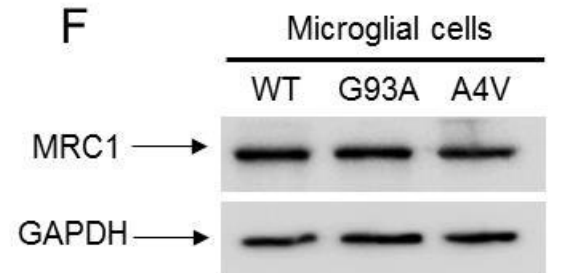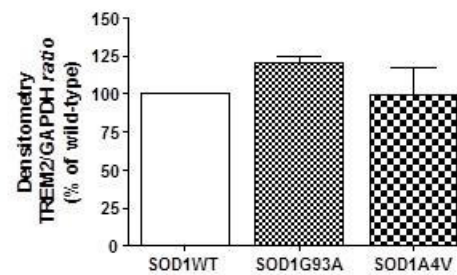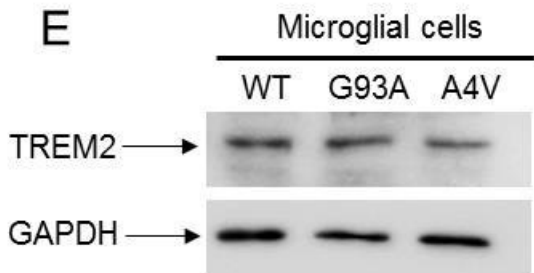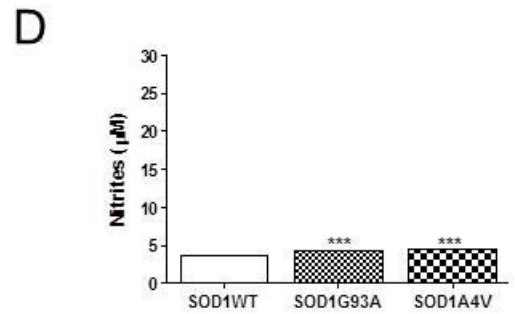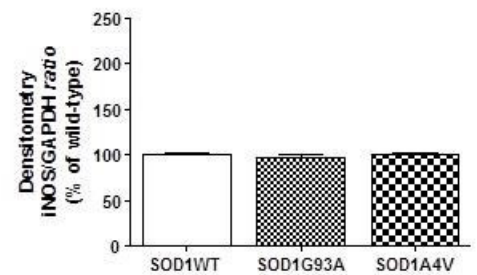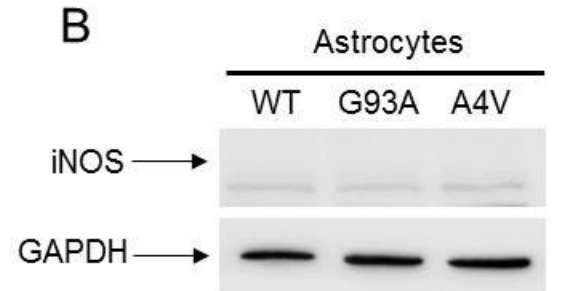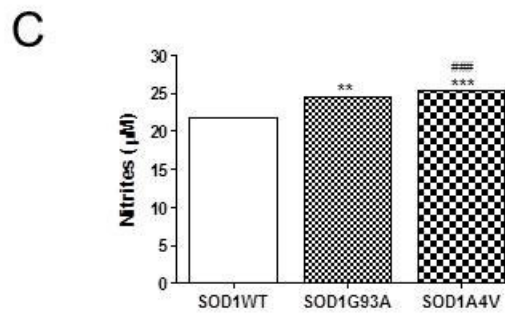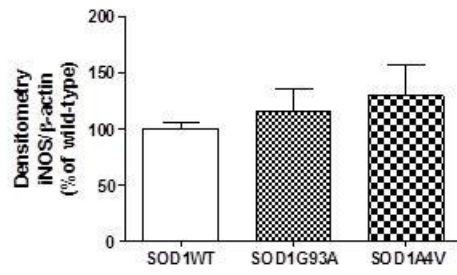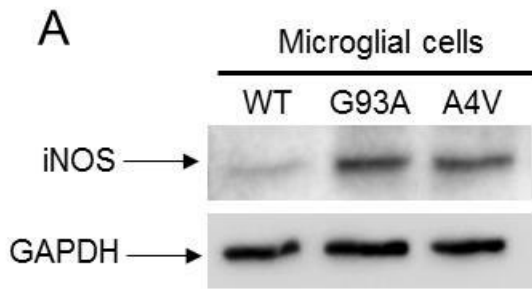
Fig.9. Effect of wild-type and mutant SOD1 overexpression on microglia and astrocyte activation, as well as on microglial M1/M2 phenotypes.

Mutant SOD1 compared to wild-type overexpression, induces partial microglial, but not astrocyte activation, as indicated by Western Blot analysis and the relative densitometry of iNOS expression, as well as by nitrite production in microglia (A, C) and astrocytes (B, D), respectively. Each bar is the mean ± S.E. of three experiments as ratio to GAPDH. Data are expressed as percentage *versus* controls. For nitrite production (C, D), each bar is the mean ± S.E. of three experiments expressed in micromolar concentration. ** $p < 0.01$, *** $p < 0.001$ relative to WT SOD1, [###] $p < 0.001$ relative to G93A SOD1. Bonferroni's post-hoc test following one-way ANOVA

4.2.5 EFFECT OF GLIAL CELLS OVEREXPRESSING WILD-TYPE OR MUTANT SOD-1 ON NEURONAL SURVIVAL/DEATH IN CONTACT CO-CULTURES

In order to clarify the role of glial cells in ALS neurodegeneration, we used an *in vitro* model of primary neuron-microglia and neuron-astrocyte contact co-cultures to evaluate the effect of wild-type and mutant SOD1 overexpression confined to glial cells, on neuronal survival and death. To this aim, we used primary differentiated cerebellar granule neurons (7DIV CGNs), since they grow as basically pure cultures without any glial cell contamination, and we then plated primary microglia or astrocytes previously transfected with SOD1-WT-EGFP, SOD1-G93A-EGFP or SOD1-A4V-EGFP on top of CGNs. We maintained co-cultures in physiological conditions or exposed them to glutamate excitotoxicity, through a chronic treatment with 100 μM glutamate for 24 h. Neuronal survival/death was evaluated by counting condensed nuclei, considered an index of apoptosis, and total nuclei, after Hoechst staining. As shown in figure 10A and 10B, differentiated CGNs co-cultured with microglia confirmed the previously demonstrated neuroprotective role of microglia against glutamate excitotoxicity (Eleuteri et al., 2008), while there was no significant difference in neuroprotection between control microglial cells and

microglial cells expressing wild-type or mutant SOD1. However, in physiological conditions, overexpression of wild-type SOD1 did not alter neuronal survival, however microglial overexpression of SOD1-G93A-EGPF and SOD1-A4V-EGFP rendered microglial cells toxic by themselves, promoting neuronal death in co-culture. On the contrary, co-culture between CGNs and astrocytes (Fig. 10C, D) evidenced a different role of astrocytes compared to microglia. Astrocytes do not seem to be neuroprotective by themselves, however overexpression of wtSOD1-EGFP in astrocytes seemed to promote neuronal survival compared to control CGNs and CGNs co-cultured with astrocytes overexpressing mutant SOD1.

A

| | CGNs | CGNs+M (SOD1WT) | CGNs+M (SOD1-G93A) | CGNs+M (SOD1-A4V) |

Control

Glut. 100μM

B

% apoptosis (condensed/total nuclei)

□ Control
▦ Glutamate 100μM

CGNs   SOD1WT   SOD1G93A   SOD1A4V

CGNs+Microglia

C

| | CGNs | CGNs+A (SOD1WT) | CGNs+A (SOD1-G93A) | CGNs+A (SOD1-A4V) |

Control

Glut. 100μM

D

% apoptosis (condensed/total nuclei)

□ Control
▦ Glutamate 100μM

CGNs   SOD1wt   SOD1G93A   SOD1A4V

CGNs+Astrocytes
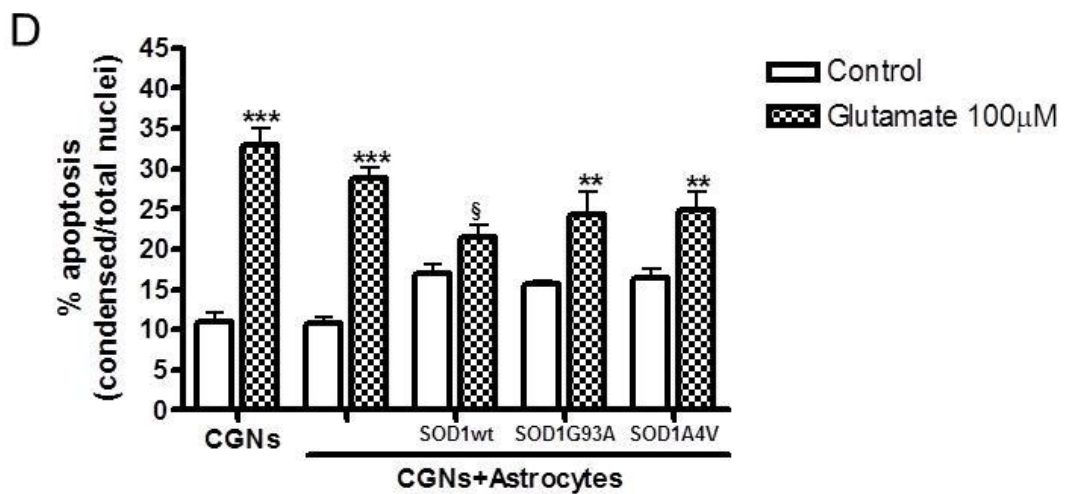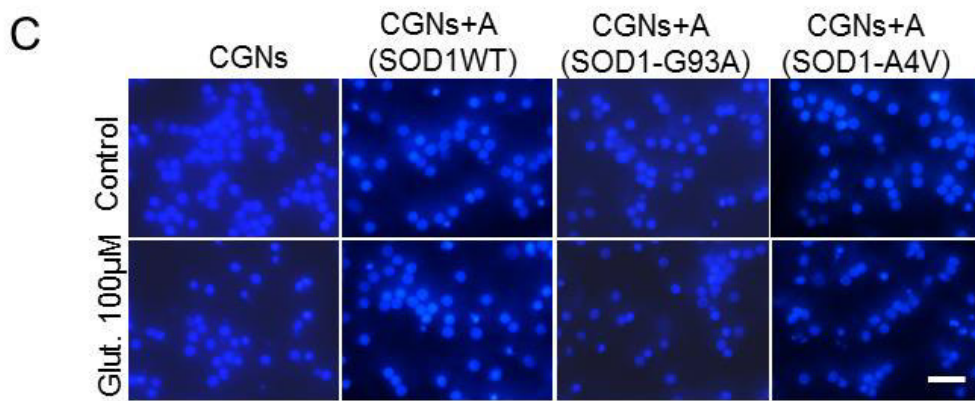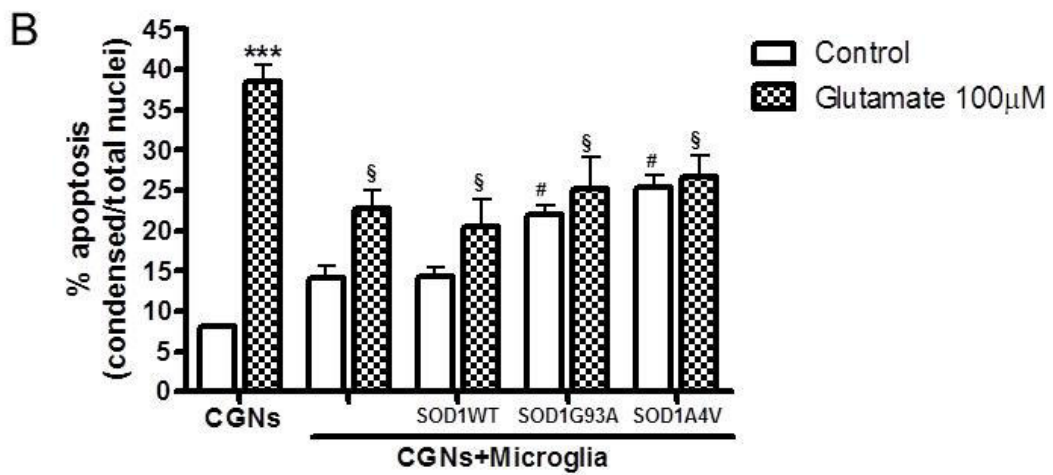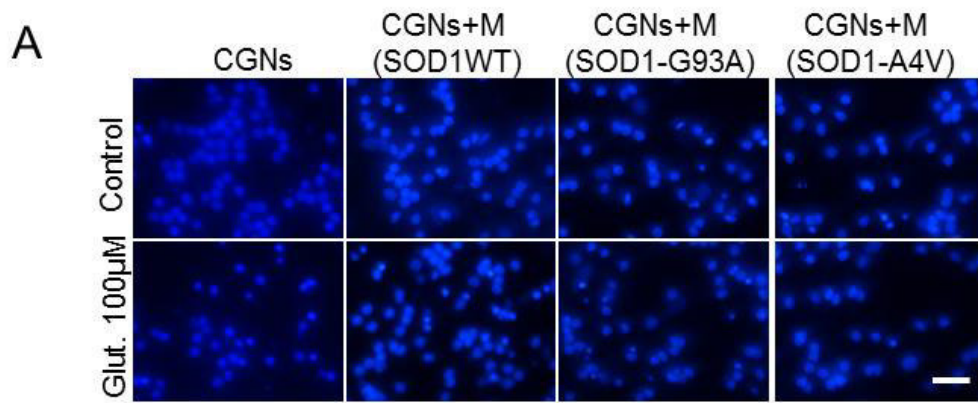
Fig.10. Effect of glial cells overexpressing wild-type or mutant SOD1 on neuronal survival/death in contact co-cultures.

Hoechst staining (A, C) and nuclei counting (B, D) of condensed/total nuclei of differentiated CGNs co-cultured with microglia (A, B) or astrocytes (C, D) overexpressing wild-type or mutant SOD1. Scale bar = 20 μm. Each bar is the mean ± S.E. of three independent experiments performed in duplicate and is expressed as the percentage of apoptosis, *i.e.* the ratio between condensed, apoptotic and total nuclei. ** $p < 0.01$, *** $p < 0.001$ relative to control CGNs; # $p < 0.01$ relative to CGNs + microglia or CGNs + astrocytes overexpressing WT SOD1; § < 0.01 relative to control CGNs + microglia or CGNS + astrocytes. Bonferroni's post-hoc test following one-way ANOVA.

4.2.6 GLUTAMATE AND SERINE RELEASE BY MICROGLIA AND ASTROCYTES OVEREXPRESSING WT AND MUTANT SOD1

Considering the role of glutamate and serine in glutamate excitotoxicity leading to ALS neuronal death (Paul and De Belleroche, 2012; 2014) and given that activated glial cells increase glutamate release (Brown and Vilalta, 2015; Milanese et al., 2009), we decided to measure the levels of glutamate and serine through HPLC in 24 h conditioned media from microglia and astrocytes overexpressing SOD1-WT-EGFP, SOD1-G93A-EGFP and SOD1-A4V-EGFP. As shown in table 1, 24h MCM contained both aminoacids at comparable levels, being glutamate ~30 μM and serine~60 μM concentrations. On the contrary, in 24 h ACM glutamate levels were lower than in 24 h ACM, though both media contained a greater amount of serine, reaching a ~ 250μM concentration. However, in media conditioned by both glial cells, no significant differences were observed following SOD1-WT-EGFP, SOD1-G93A-EGFP or SOD1-A4V-EGFP over-expression, thus indicating that the presence of mutant SOD1 alters the release of SOD1 by itself, as well as of other proteins (Basso et al., 2013), but not the release of these two important aminoacids, at least in our experimental conditions.

95

	Concentration (µM)	Control	SOD1-WT	SOD1-G93A	SOD1-A4V
microglia	**glutamate**	39,53 ±13,81	32,88±10,70	33,85±10,27	47,43±9,62
	serine	79,73±17,54	61,95±18,54	59,55±15,96	68,63±21,83
astrocytes	**glutamate**	11,63±6,21	12,43±2,11	12,03±1,99	10,60±1,66
	serine	189,97±36,16	272,73±72,15	249,97±64,90	312,93±21,53

Table 1. HPLC measurement of glutamate and serine in 24 h conditioned media.

HPLC measurement of glutamate and serine concentrations in 24h conditioned media from primary rat microglia and astrocytes overexpressing EGFP-SOD1-WT or EGFP-SOD1-G93A or EGFP-SOD1-A4V. The table shows the µM concentration of each amino acid. Each value is the average of three / four experiments ± SE. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.2.7 TREHALOSE REDUCES THE INTRACELLULAR ACCUMMULATION OF SOD1

Trehalose (a-D-glucopyranosyl a-D-glucopyranoside) is a disaccharide naturally occurring in insects, plants, fungi and bacteria, though not in vertebrates (Richards et al., 2002). Trehalose protects against environmental stress, desiccation, dehydration, heat, cold and oxidation (Richards et al., 2002; Chen and Haddad, 2004). It was reported that trehalose can promote the clearance of autophagy substrates, such as mutant huntingtin protein or the A30P and A53T mutants of alpha-synuclein (Sarkar et al., 2007). Oral administration of trehalose in a transgenic mouse model of HD decreased polyglutamine aggregates in the brain and liver, improved motor function and extended lifespan (Tanaka et al., 2004). It has also been reported that trehalose treatment can ameliorate behavioral and pathological symptoms in a mouse model of tauopathy (Rodriguez-Navarro et al., 2010). Considering the data previously obtained on intracellular mutant SOD1 accumulation and the

preliminary data on autophagy impairment in primary microglial cells, we decided to study whether this treatment could promote the clearance of mutant SOD1. As previously described, we transiently overexpressed in rat primary cultures of microglia and astrocytes wild-type SOD1, G93A SOD1and A4V SOD1 fused with the GFP reporter gene. Two hours after transfection, cells were treated with 5 mM trehalose for 48 hour in serum free BME to improve the autophagic flux. Western blot analysis performed on media conditioned for 48 h after transfection (Fig.11A) showed that both wild-type and mutant SOD1 were released by microglia with a significant difference in SOD1 release between cells that overexpressed the two SOD1 mutants and wild-type SOD1 without any difference between control cells and treated cells. Interestingly, Western blot analysis performed on the same cells from which we collected the previously shown conditioned media (Fig.11C) showed that mutant SOD1 levels were significantly decreased by trehalose treatment suggesting that trehalose enhances autophagy activity and accelerates the degradation of mutant SOD1.
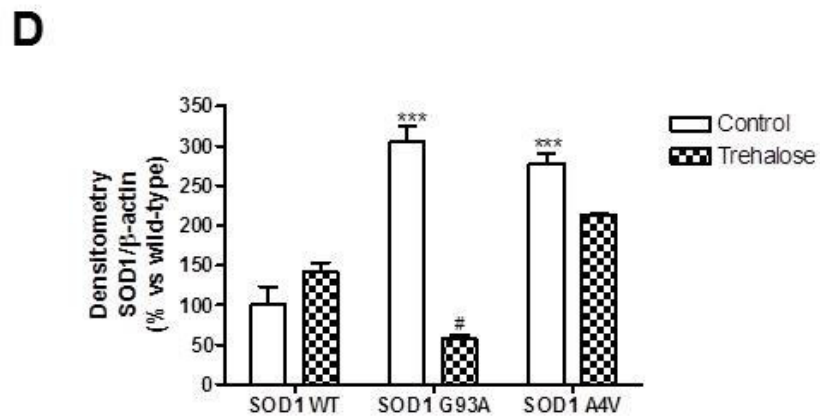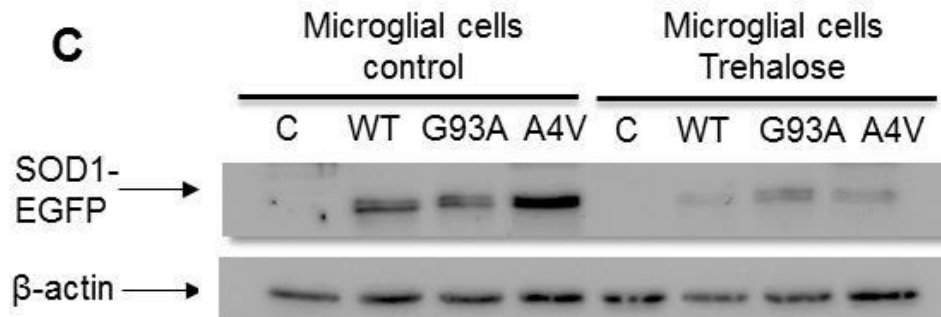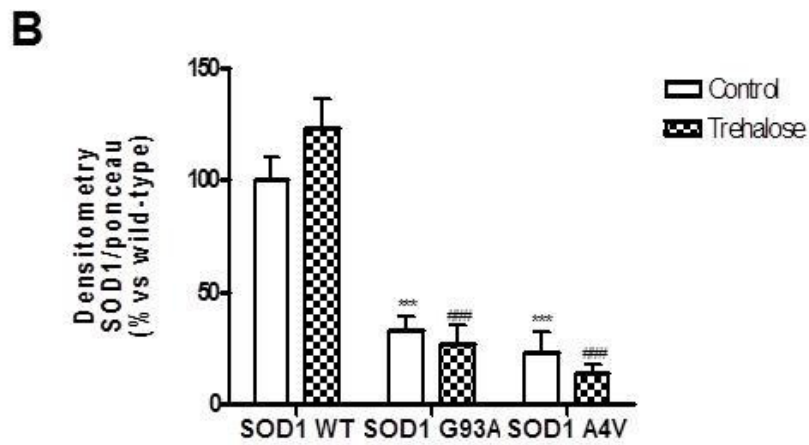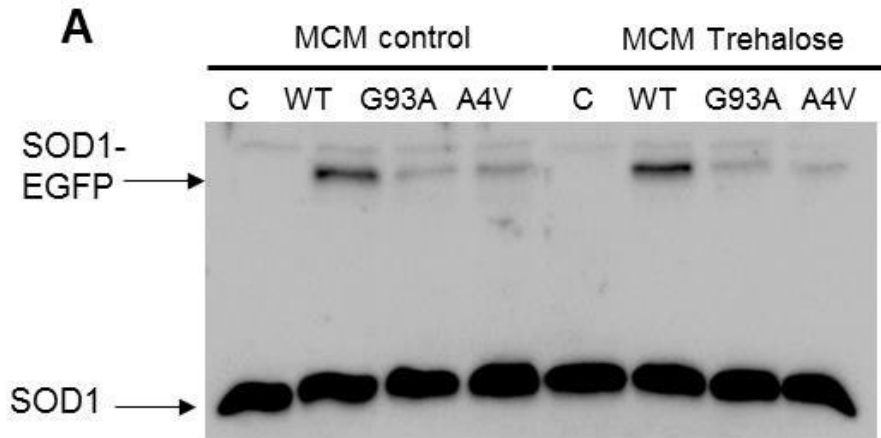
Fig.11. Trehalose restores mutant SOD1 expression in microglia similar to control levels.

Western blot analysis and relative densitometry of SOD1 released (A) and expressed (C) in 48h conditioned medium and in lysed microglial cells overexpressing wild-type SOD1, G93A SOD1 and A4V SOD1 with or without trehalose treatment. Each bar represents the percentage *versus* SOD1-WT of exogenous/endogenous SOD1 ratio for conditioned media and of SOD1/β-actin ratio in cell lysates. Each bar represents the mean ± S.E. of four samples from different experiments. *** $p < 0.001$ compared to WT SOD1; # $p < 0.05$, ###$p < 0.001$ compared to G93A SOD1. Bonferroni's post-hoc test following one-way ANOVA.

4.2.8 TREHALOSE REDUCES THE ACTIVATION STATE OF MICROGLIAL CELLS OVEREXPRESSING MUTANT SOD1

Protein intracellular accumulation, observed in many proteinopathies, seems to be responsible for glial cell activation (Ferretti et al., 2012), defined by the increase in the intracellular expression of iNOS and the consequent release of NO (Yuste et al., 2015). As previously showed (Fig.9 A,C), mutant SOD1 overexpression promoted an activation state in microglial cells without any change in the expression of two markers related to the alternative microglial activation, TREM2 and MRC1, thus suggesting that in our experimental conditions microglial cells did not lose the M2 features. Data recently published, showed an interesting reduction in CD11b levels in the spinal cord of trehalose-treated G93A SOD1 mice compared to controls, as well as a reduction of IBA1 and GFAP levels in the lumbar spinal cord of the same animals at both 90 days of age and during end-stage disease (Li, 2015), indicating these conditions as evidence of a reduced gliosis that occurs in ALS mouse models and patients. Trehalose treatment of primary microglia overexpressing wild-type SOD1, G93A SOD1 and A4V SOD1, reduced the intracellular expression of iNOS (Fig. 12B) without interfering with microglial phenotype (Fig.12C).
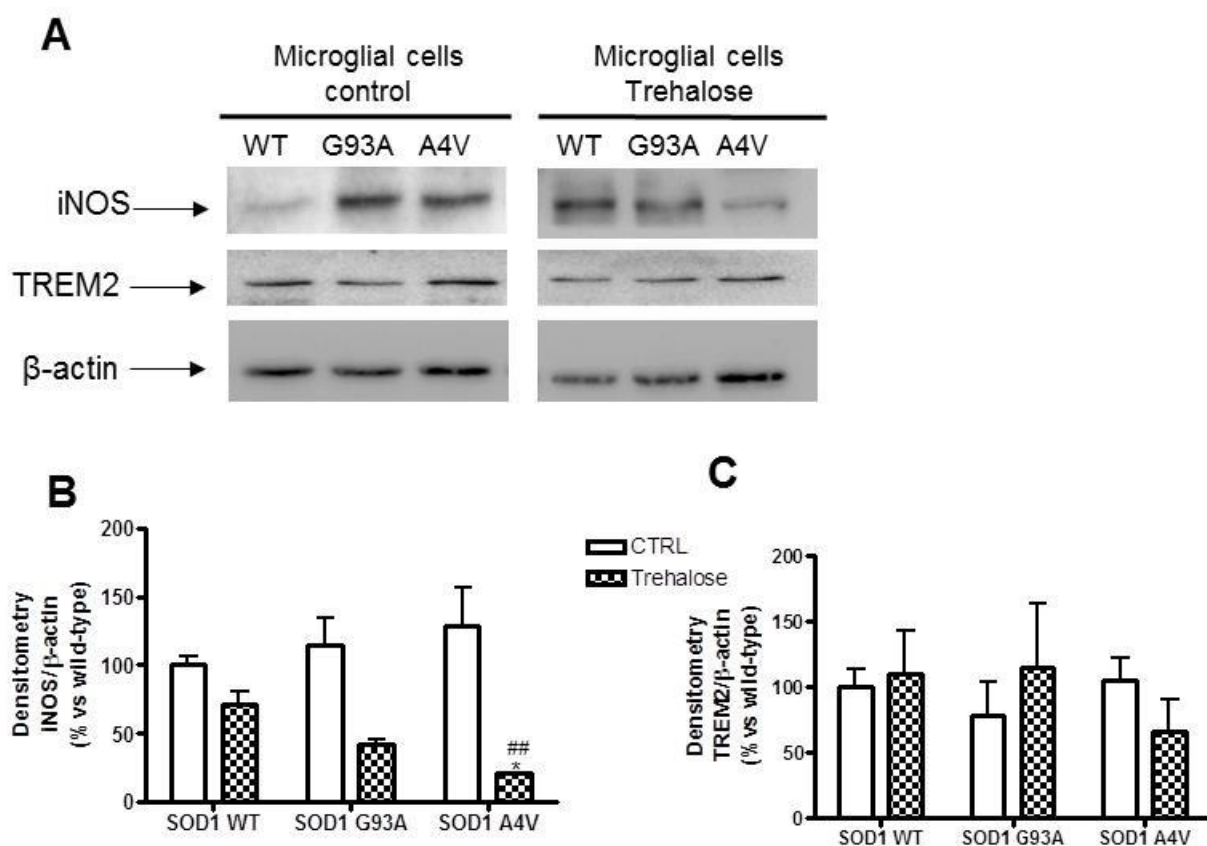
Fig.12. Effect of trehalose treatment on microglial activation.

Western blot analysis and relative densitometry of iNOS and TREM2 in microglial cells overexpressing wild-type SOD1, G93A SOD1 and A4V SOD1 for 48h with or without 5 mM trehalose treatment. Each bar represents SOD1/β-actin ratio. Each bar represents the mean ± S.E. of four samples from different experiments. * $p < 0.05$ compared to WT SOD1; ## $p < 0.01$ compared to G93A SOD1. Bonferroni's post-hoc test following one-way ANOVA.

4.2.9 NEUROPROTECTIVE FUNCTION OF TREHALOSE IN GLIA-NEURON CO-CULTURE MODEL

Co-culture is an easy *in vitro* model to mimic the crosstalk between glial cells and neurons. As previously described, microglia exerted a neuroprotective effect in cerebellar granule cells exposed to glutamate, but microglial overexpression of mutant SOD1 appears to be toxic to neurons.

Astrocytes, on the other hand, did not show any neuroprotective effect by themselves, though they seemed to display a neuroprotective role when wild-type SOD1 was overexpressed. According to the "non-cell-autonomous mechanisms" and considering the ability of toxic molecules to spread to neighbouring cells promoting injury as well as the previous promising results on the efficacy of trehalose in removing accumulated SOD1 in microglia, we performed a co-culture model between microglia or astrocytes overexpressing SOD1-WT-EGFP, SOD1-G93A-EGFP or SOD1-A4V-EGFP and differentiated primary cerebellar granule neurons. Both types of co-cultures were exposed to treatment with 100 mM glutamate for 24h, which mimics excitotoxicity or with 5 mM trehalose or with both trehalose plus glutamate. As previously reported, glutamate is toxic for neurons, since CGNs showed a significant increase in apoptosis percentage compared to control cells. Microglia, known to be neuroprotective in case of neuronal damage, in control conditions or overexpressing wild-type SOD1, partially reduced glutamate toxicity, but the simultaneous treatment with trehalose surprisingly restored and even decreased neuronal death under control conditions. Microglial overexpression of SOD1-G93A-EGPF and SOD1-A4V-EGFP rendered microglial cells toxic by themselves, promoting neuronal death in co-cultures. The promising ability of trehalose is not only reducing neuronal death but, even more significantly to our research, it seems to decrease microglia toxicity due to mutant SOD1 overexpression.
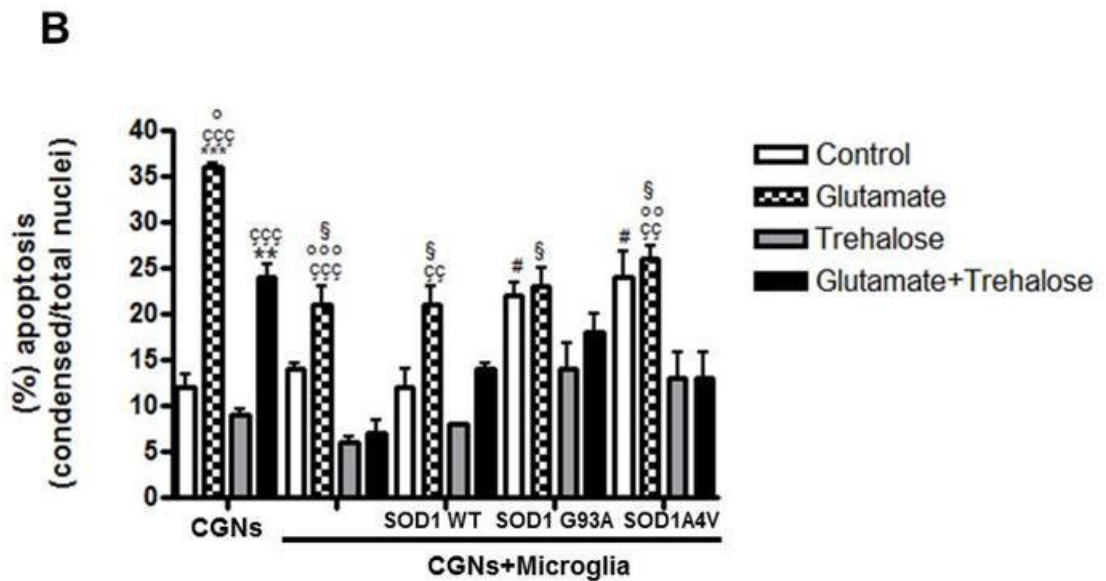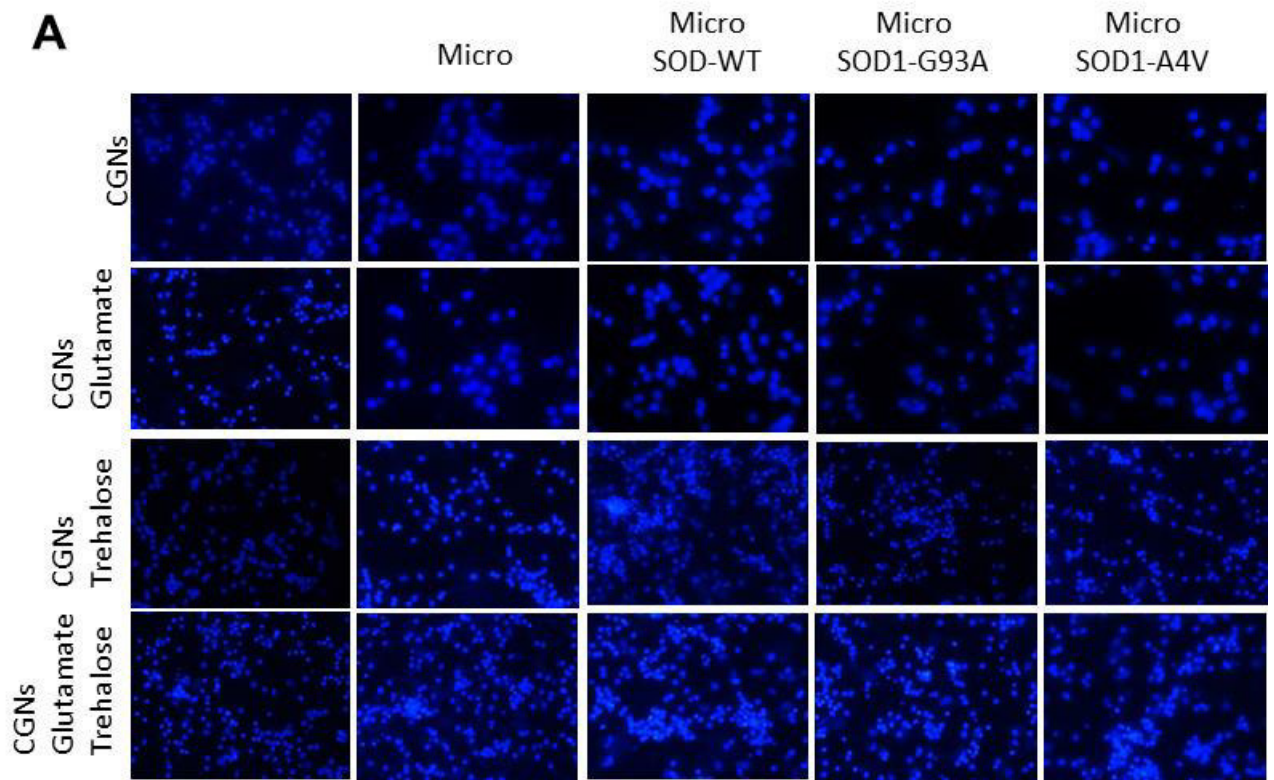
Fig.13. Effect of trehalose on microglial neuroprotective/neurotoxic effects due to overexpression of wild-type or mutant SOD1 in co-cultures with CGNs

Hoechst staining (A) and nuclei counting (B) of condensed/total nuclei of differentiated CGNs co-cultured with microglia overexpressing wild-type or mutated SOD1 in presence or absence of threalose. Scale bar = 20 μm. Each bar is the mean ± S.E. of three independent experiments performed in duplicate

and expressed as the percentage of apoptosis, *i.e.* the ratio between condensed, apoptotic and total nuclei. ** $p < 0.01$, *** $p < 0.001$ relative to control CGNs; #$p < 0.01$ relative to CGNs + micro overexpressing WT SOD1; § < 0.01 relative to control CGNs + microglia; ° $p < 0.05$; °° $p < 0.01$; °°° $p < 0.001$ relative to the respective Glutamate + Trehalose treatment; ^{çç} $p < 0.01$; ^{ççç} $p < 0.001$. Bonferroni's post-hoc test following one-way ANOVA.

4.3 RELEASE OF ALPHA-SYNUCLEIN BY GLIAL CELL LINES IN PHYSIOPATHOLOGICAL CONDITIONS.

4.3.1 α-SYNUCLEIN EXPRESSION AND RELEASE FROM RAT PRIMARY CULTURES OF MICROGLIA, ASTROCYTES AND DIFFERENTIATED CGNS

We have previously shown results on the release of two proteins, PNP and SOD1, respectively involved in neuropathological conditions such as PNP-deficiency and ALS, in primary rat cultures of microglia and astrocytes. Considering the role of many proteins in the onset of neurodegenerative diseases and the well-known proteinophaties, we tried to define the release of α-synuclein by glial cells both in physiological and pathological conditions, i.e. Parkinson's disease.

α-synuclein (α-syn) is involved in many neurodegenerative diseases, including Parkinson's disease (PD) where the protein is the main component of toxic aggregates called "Lewy bodies" (Steiner et al., 2011; Kahle, 2008). Although α-synuclein function in cells is not yet clarified, according to the well-known hypothesis, the protective or toxic effect of α-synuclein depends on its expression levels. α-synuclein under physiological conditions would have a protective function, while its accumulation and its aggregation into oligomers or fibrils would have a detrimental effect for the cell (Irwin et al., 2013; Vekrellis et al., 2004). It is known from the literature that α-synuclein is

expressed and secreted by neurons, including cerebellar granule cells, but it is unknown whether it can also be released by microglia and astrocytes (Lee et al., 2010).

A first aim of this study was to evaluate the gene expression of SNCA (encoding the α-synuclein protein in Rattus norvegicus) by Quantitative Real Time PCR (qRT-PCR) on primary cultures of cerebellar granule neurons, microglia and astrocytes. Cerebellum granules are known to contain high levels of α-synuclein. As shown in Table 2, SNCA mRNA levels are 50 times higher in cerebellar granules compared to astrocytes and 2500 times higher than microglia.

	α-syn media Ct	GAPDH media Ct	dCt α-syn – GAPDH	ddCt dCt cell type – dCt granuli	Valori assoluti rapporto α-syn rispetto ai granuli	
Microglia	42,77 ± 0,76	26,93 ± 0,87	15,83 ± 1,05	11,67 ± 0,89	0,0004	(0,0001-0,0006)
Astrociti	36,52 ± 0,24	27,13 ± 0,67	10,6 ± 0,30	6,43 ± 0,99	0,0188	(0,005-0,044)
Granuli	26,70 ± 1,15	26,55 ± 0,63	4,16 ± 1,12	0	1	

Table 2. SCNA mRNA expression and qRT-PCR quantification

Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as the difference between the target gene and the expression of the endogenous gene β-actin. Values are the mean ± SE of 3 independent experiments.

Lee and colleagues have shown that α-synuclein is transmitted from neuron to neuron and from neuron to astrocyte through endocytosis and inclusion bodies (Rappold et al., 2010; Lee et al., 2010), but whether it may also be released by glial cells has not been yet clarified. Thus, we examined the expression and release of α-synuclein in primary rat cultures of microglia and astrocytes. Primary cultures of astrocytes and microglia were cultured in serum-free BME, cells were then lysed and media collected after 2, 24 and 48

hours. α-synuclein expression in cells and release in media were analyzed by Western blot. As shown in Figure 12 A, C, α-synuclein is expressed in both cell types, especially in microglia where it decreases over time (Fig. 14A). At the same time, α-synuclein is constitutively secreted by microglial cells and accumulates in the conditioned medium (Fig. 14C). In astrocytes, a peak of expression occurs at 24h (Fig. 14B), while the Western blot relative to conditioned media from astrocytes does not show secretion of α-synuclein over time (Fig.14D).
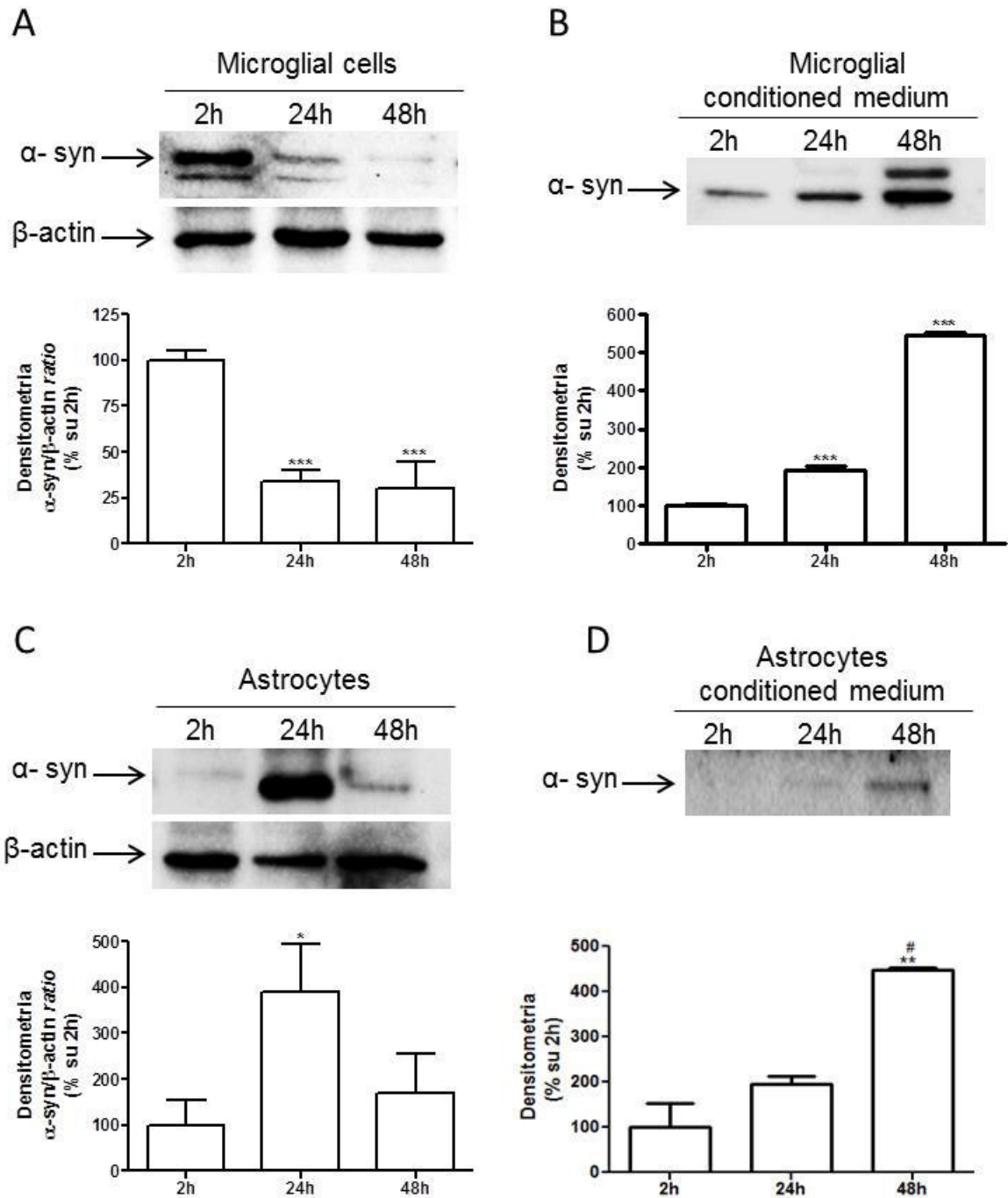
Fig. 14. α-synuclein expression in rat primary microglia, astrocyte and cerebellar granule neuron (CGNs) cultures.

Western blot analysis and relative densitometry of α-syn expression in microglia (A), astrocytes (C) and the relative release in conditioned medium from microglia (B) and astrocytes (D). Each bar represents the mean ± SE of 3 independent experiments as the ratio to β-actin. 500 µl of conditioned medium

were concentrated and loaded to assay α-synuclein release. Data are expressed as percentage to controls. * p < 0.05; ** p < 0.01 compared to controls. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test. Equal loading was confirmed by staining with Red Ponceaus S performed on transfered membranes.

4.3.2 α-SYNUCLEIN EXPRESSION IN MURINE IMMORTALIZED CELL LINE OF MICROGLIA (N9) AND ASTROCYTES (IMA2.1)

Since the aim of this study was to analize the expression and release of α-synuclein by glial cells, in order to limit the use of animals, we decided to assess whether the physiological expression and release of α-synuclein could take place in murine immortalized cell lines of astrocytes (IMA2.1) and microglia (N9).

Both cell lines were cultured in serum free media, as done for primary cultures, in order to define a similar α-synuclein expression between primary and immortalized lines necessary to understand whether immortalized cells could be a reliable model for our studies. Western blot analysis of α-synuclein expression in both cell lines (Fig. 15 A and B), showed a progressive decrease over time, suggesting that α-synuclein may be mainly released.

We also performed immunofluorescence staining to observe the intracellular localization of α-synuclein related to the lysosomal marker LAMP-1. Comparing the immunofluorescence staining on primary astrocytes to murine cell lines (Fig. 15 C), a cytoplasmic α-synuclein "spot" localization was observed, being more evident in primary astrocytes which may indicate a localization in secretory granules, probably of lysosomal nature, confirmed by partial co-localization with the LAMP-1 marker. Partial co-localization with LAMP-1, could indicate that at least part of α-synuclein is exchanged between cells using vesicles (Takenouchi et al., 2009).
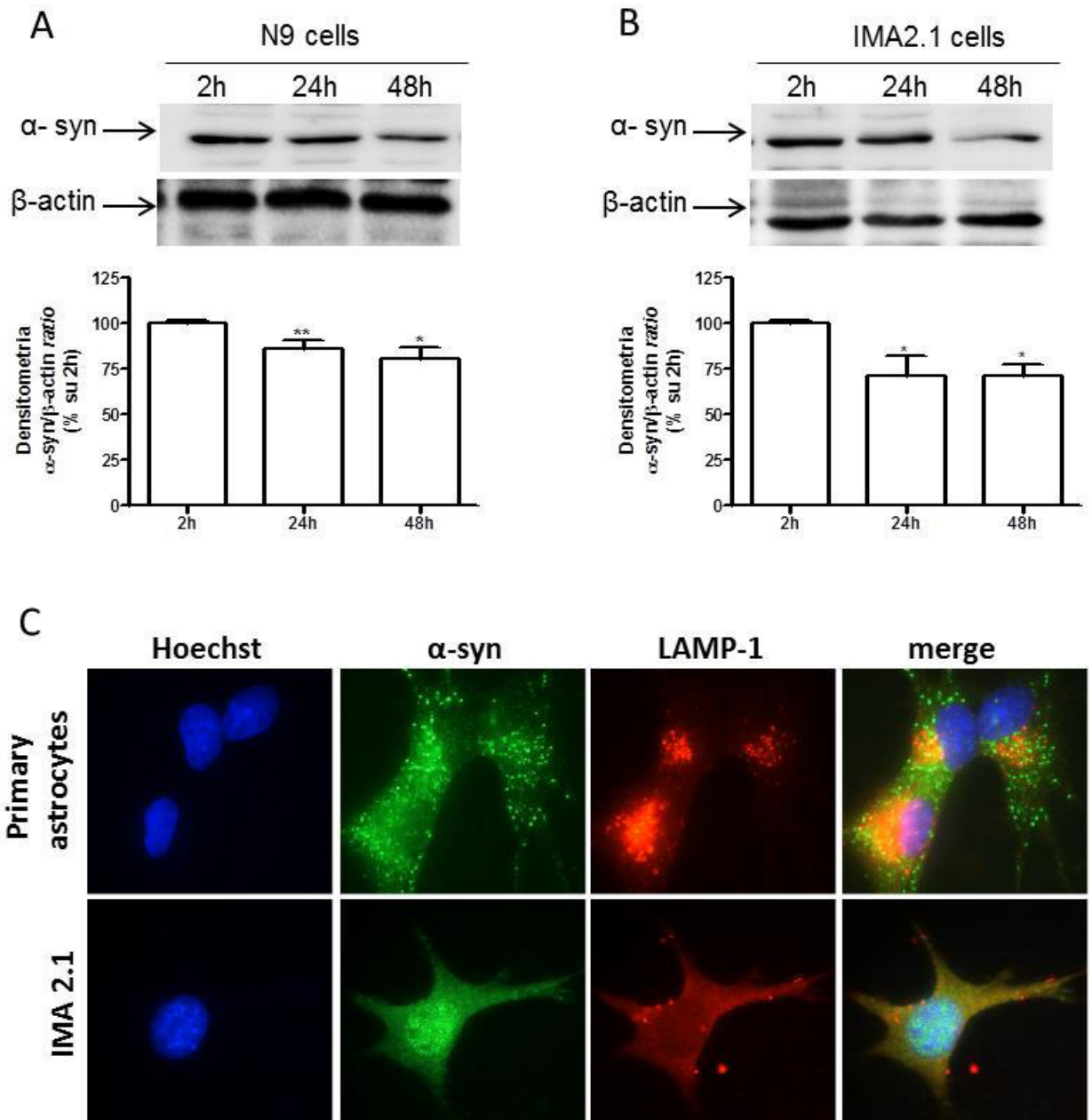
Fig. 15. α-synuclein expression in immortalized cell lines of microglia (N9) and astrocytes (IMA2.1).

Western Blot analysis and relative densitometry of cell lysates from N9 (A) and IMA 2.1 cells (B) show that α-synuclein expression gradually decreases over time in both cell lines. Each bar represents the ratio between α-syn compared to β-actin. Each bar represents the mean ± SE from 3 experimets expressed as percentage to control. * p < 0.05; ** p < 0.01, compared to

control (α-synuclein at 2h). (C) Immunocytochemical analysis of α-synuclein (green) localization related to LAMP-1 (red).

4.3.3 α-SYNUCLEIN RELEASE IS MEDIATED BY LYSOSOMAL SECRETORY PATHWAY

Since glial secretion through extracellular vesicles (EVs) is has become increasingly important in physiological glial-neuron communication, we assessed whether α-synuclein could be released from glial cells through this pathway. EVs were isolated from conditioned medium from IMA 2.1 and N9 cells cultured for 2 hours in serum free medium. Western blot analysis revealed the presence of α-synuclein in conditioned media as shown in Fig. 14 A, B, where it accumulates over time. α-synuclein was also present in the pellet fraction containing vesicles, positive to LAMP-1. These data demonstrate that microglia and astrocytes and the respective immortalized cell lines release α-synuclein through both a classical and an EVs-mediated release, the latter occurring through the lysosomal secretory pathway.
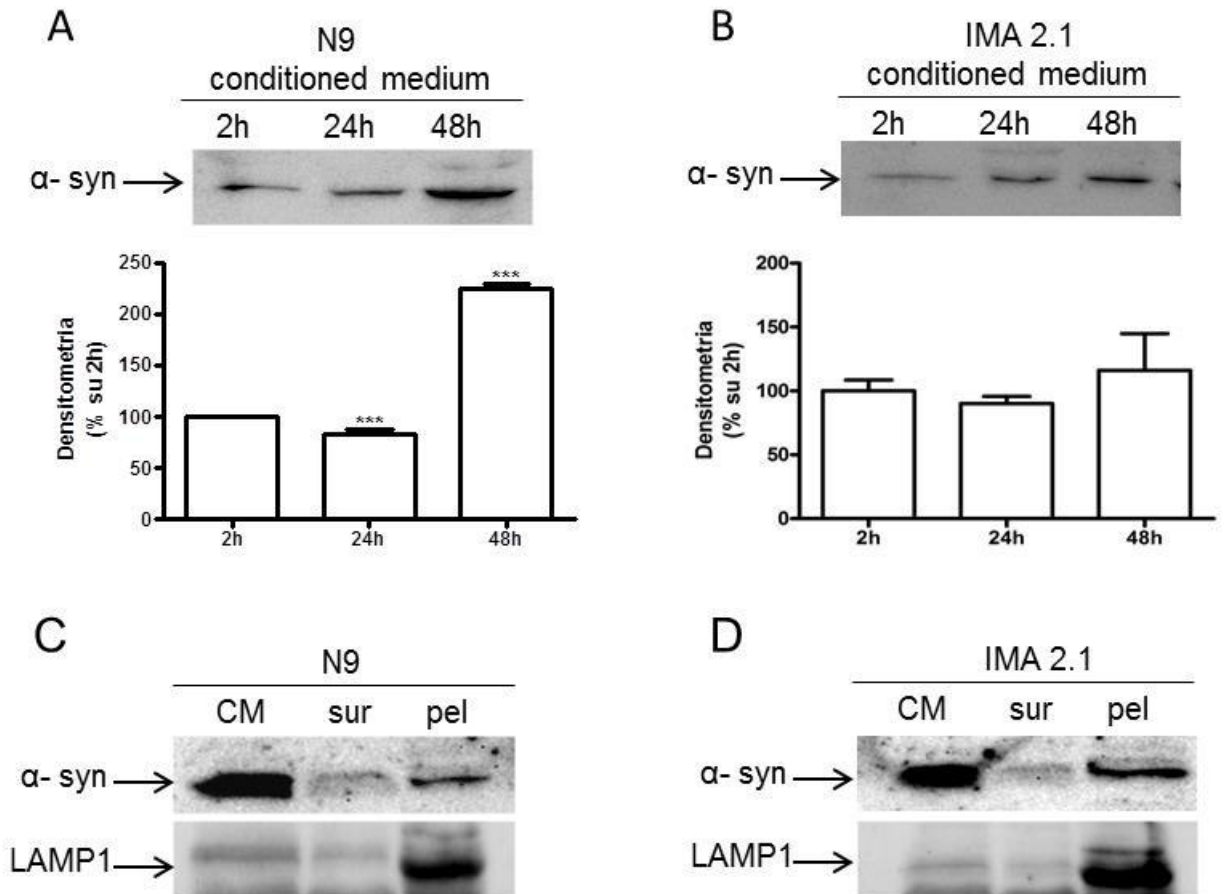
Fig. 16. α-synuclein release via extracellular vesicles (EVs).

Western blot analysis of conditioned medium shows constitutive secretion of α-synuclein by both N9 and IMA 2.1 cell lines (A,B). Ultracentrifugation of conditioned media from N9 microglia (C) and IMA 2.1 astrocytes (D) shows α-synuclein release even through vesicles. Each bar represents the mean percentage ± SE of 3 samples from independent experiments; * $p < 0.05$; *** $p < 0.001$ compared to control (α-synuclein at 2h). One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.3.4 EFFECT OF INFLAMMATORY STIMULI ON α-SYNUCLEIN RELEASE

Previous results showed constitutive expression and secretion of α-synuclein from microglia and astrocytes. We decided to investigate whether and how constitutive expression and secretion could be modulated in response to inflammatory stimuli able to activate IMA 2.1 and N9 cell lines. If on one hand the primary activation of microglia and astrocytes reflect a physiological mechanism leading to neuroprotection (Hu et al., 2015; Molofsky et al., 2012), on the other hand overactivation or the loss of glial cell functions may increase a pre-existing neuropathology (Streit et Xue, 2009).

The first method used for microglial activation described in the literature was treatment with the bacterial endotoxin lipopolysaccharide (LPS) (Hetier et al., 1988). IMA 2.1 and N9 cells were treated for 24 hours in DMEM medium without serum with increasing concentration of LPS [0, 10, 100 and 1000 ng / ml]. As observed, the expression of α-synuclein in both cell types remained almost unchanged after treatment with different concentrations of LPS (Figure 17.1 A, B), while its secretion increased in proportion to the concentration of LPS (Fig. 17.1 C, D). Simultaneously to α-synuclein, the expression of inducible nitric oxide synthase (iNOS) and nitrite release in the medium were studied as an indicator of activated glia. Treatment of N9 cells with increasing concentration of LPS increased the expression of iNOS in a dose dependent way (Fig. 17.2 E), while in IMA 2.1 cells only the 100 ng/ml LPS treatment increased the expression of iNOS, while the highest LPS concentration may be toxic for these cells (Fig. 15.2 F). In both cell types, LPS treatment promoted not only an increase in α-synuclein release but also an increase in nitrite release in the medium (Fig. 15.2 G, H).
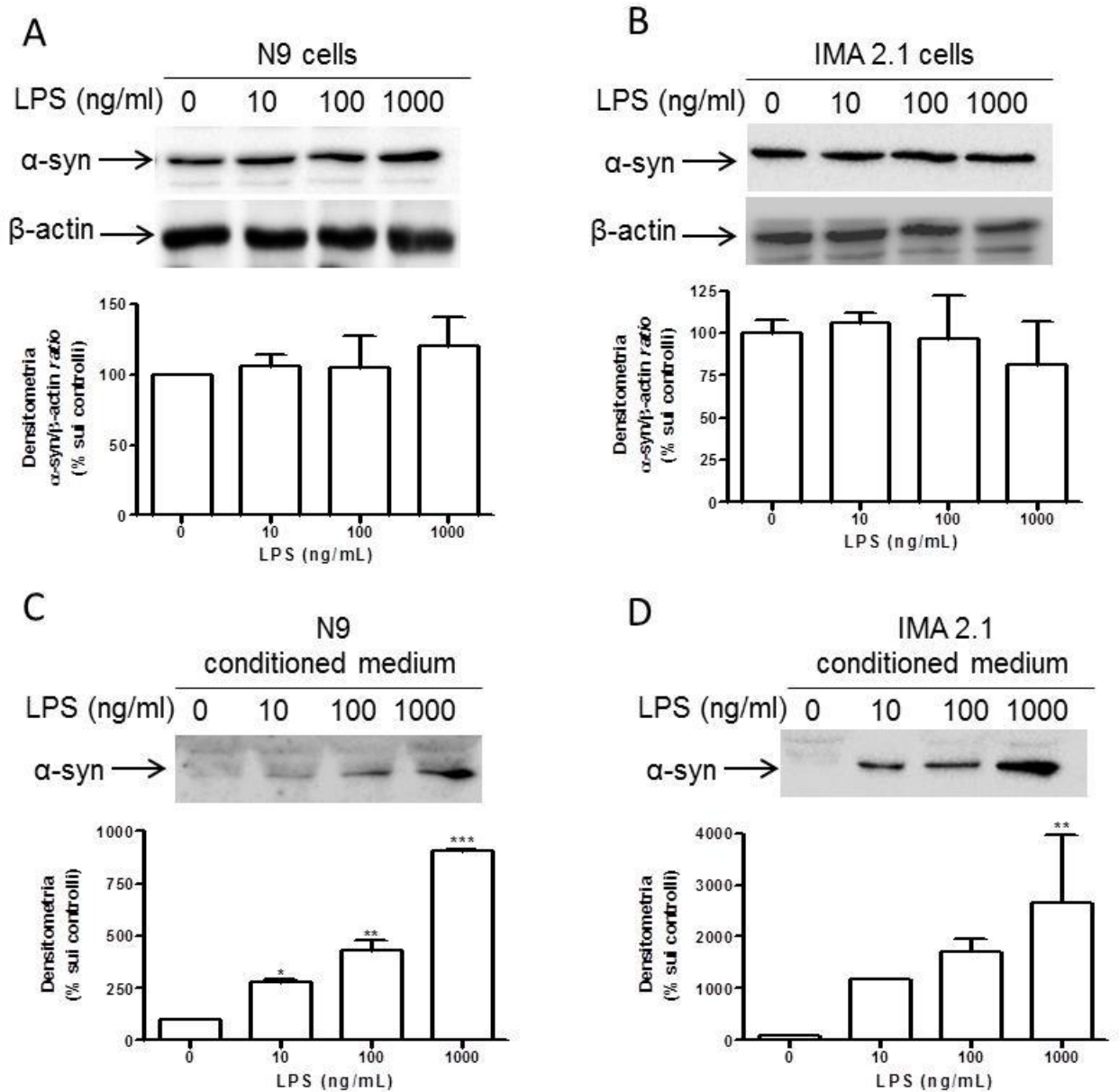
Fig 17.1. Effect of lypopolyshaccharide (LPS) on α-synuclein release by microglia and astrocytes.

Representative western blot analysis and densitometry of α-synuclein expression in N9 (A) and IMA2.1 cells (B) and release from N9 (C) and IMA2.1 cells (D) stimulated for 24 hours with increasing LPS concentrations [1; 10; 100; 1000 ng/ml]. Each bar represents the mean ± SE of 3 samples from independent experiments as the ratio to β-actin related to the control. * p <

0.05; ** p < 0.01, *** p < 0.001 compared to the control. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.
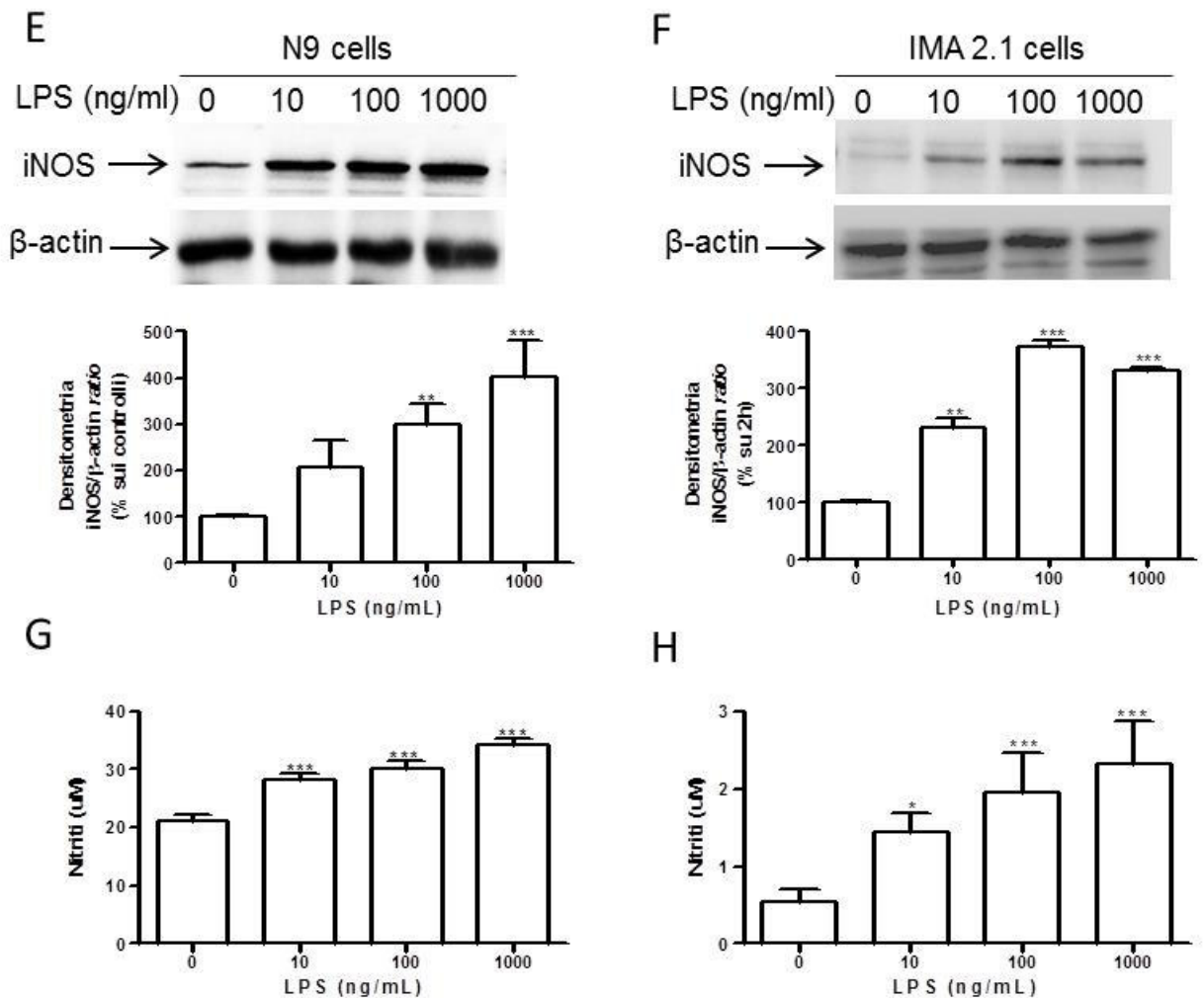
Fig.17.2. Effect of inflammation on microglia and astrocyte activation.

Western blot analysis and relative densitometry of N9 (E) and IMA 2.1 cells (F) show increased activation (iNOS expression) depending on LPS doses. Each bar represents the ratio of iNOS compared to β-actin. Together with an iNOS increase, nitrite secretion in the medium from N9 (G) and IMA 2.1 cells (H) was also increased. Each bar represents the mean percentage ± SE of 3 samples; * p < 0.05; ** p < 0.01; *** p < 0.001 compared to the control. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.3.5 GLIAL ACTIVATION THROUGH ATP DOES NOT CHANGE α-SYNUCLEIN RELEASE

To test the effect of physiological-like glial activation, we treated IMA 2.1 and N9 cell lines for 4 hours with increasing concentrations of ATP [0, 100 and 500 uM] in serum free DMEM medium.

By increasing ATP concentrations, α-synuclein expression remains almost unchanged in the N9 microglia cell line (Fig.18.1A) and IMA 2.1 astrocyte cell line (Fig.18.1B). ATP treatment seems to slightly increase, but not in statistically significant way, α-synuclein release from microglia (Fig.18.1C,) while it does not affect α-synuclein release by astrocytes (Fig.18.1D). Furthermore, the highest ATP concentration [500 uM] appears to activate N9 cells, as evidenced by the increased expression of iNOS (Fig. 18.2 E) and nitrite secretion (Fig. 18.2 G), while IMA 2.1 cells do not seem to be affected by ATP treatment (Fig. 18.2 F,H).
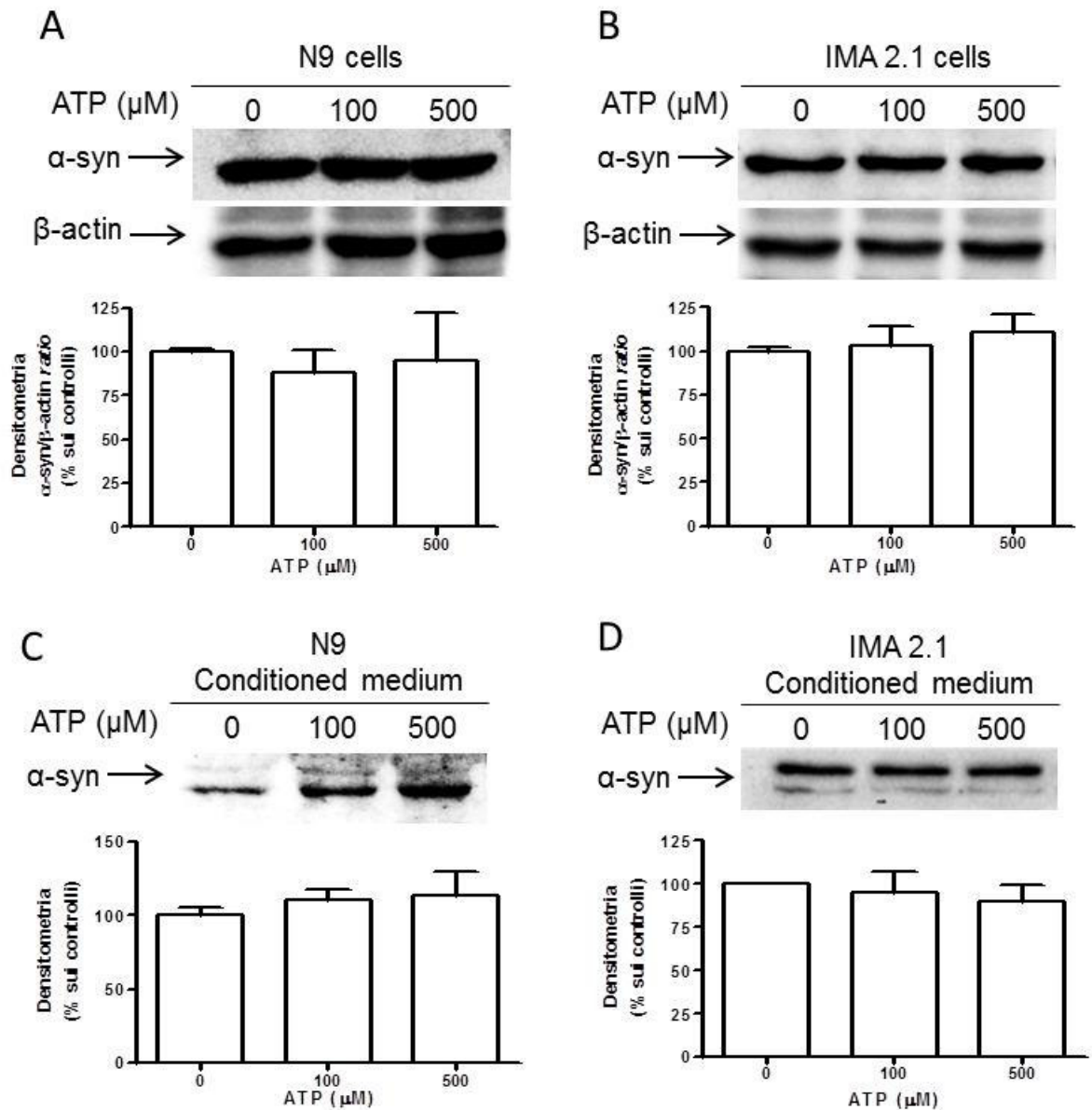
Fig.18.1. Effect of ATP treatment on α-synuclein release by microglia and astrocytes.

Western Blot analysis and relative densitometry of α-synuclein expression (A,B) and release in 4-hour conditioned media (C,D) from N9 (A,C) and IMA 2.1 (B,D) cells treated with increasing ATP concentrations [0, 0,5 or 1 mM]. Each bar represents the mean ± S.E of 3 independent samples as the ratio to β-actin. 500µl of conditioned medium from each sample were loaded. Each bar represents the mean ± SE of 3 samples from independent

experiments compared to the control. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.
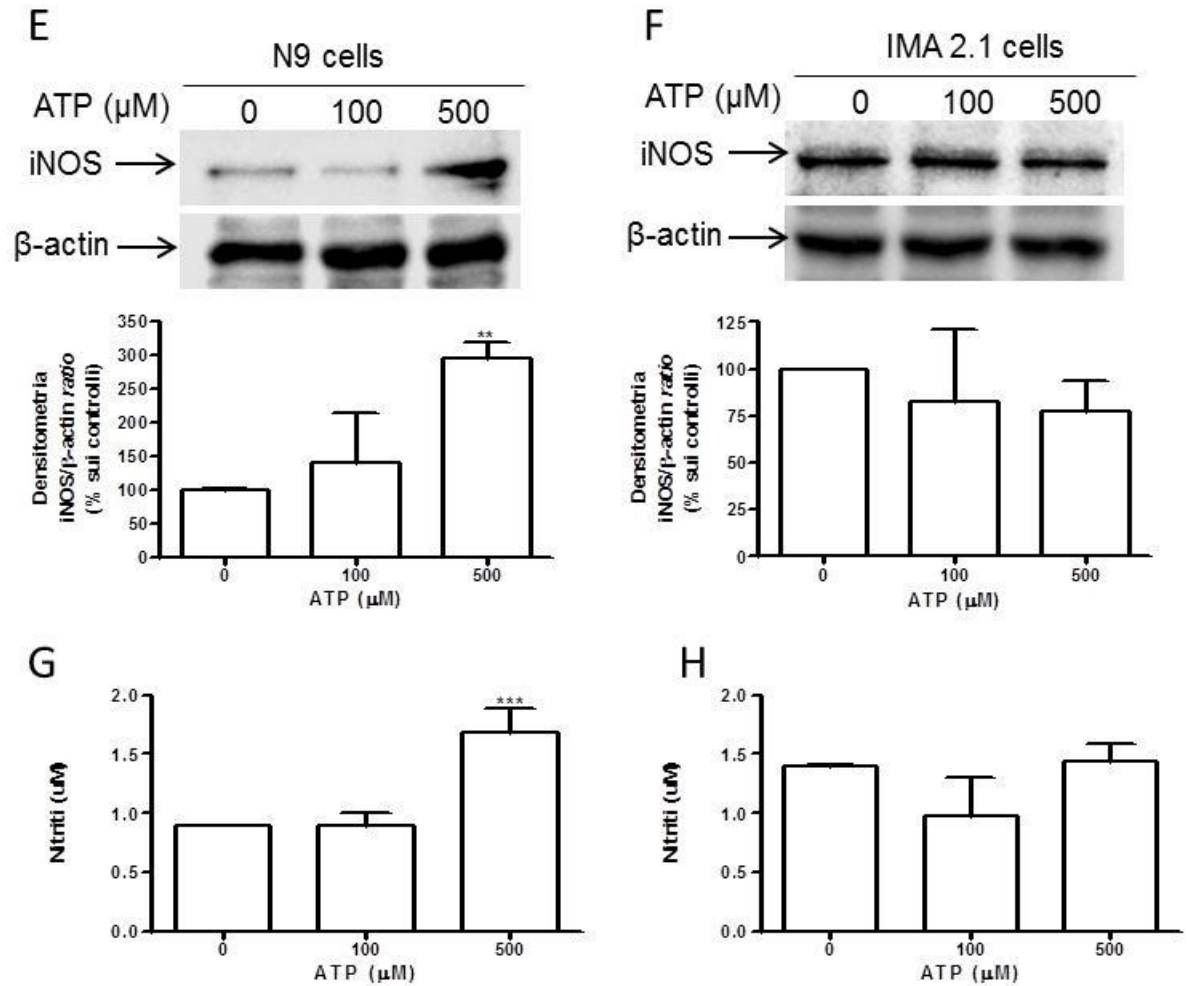
Fig.18.2. Effect of P2X7 receptor activation on microglia and astrocyte activation.

Western blot analysis and relative densitometriy on lysates from N9 (E) and IMA 2.1 cells (F) shows the increased activation (iNOS expression) depending on ATP doses in microglial cells (E) but not in IMA2.1 cells (F). Each bar represents the ratio of iNOS compared to actin. Nitrite secretion in the medium shows the same trend as iNOS activation (G) (H). Each bar represents the mean percentage $\pm$ SE of 3 samples; ** $p < 0.01$; *** $p < 0.001$ compared to the control. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.3.6 ACTIVATION OF NMDA RECEPTOR MEDIATED BY SERINE DOES NOT INTERFERE WITH PHYSIOLOGICAL GLIAL RELEASE OF α-SYNUCLEIN

D-serine is a neuromodulator of N-methyl-D-aspartate receptors (NMDAR), able co-activate these receptors when they bind glutamate. The NMDA receptor has a binding site for glycine, however D-serine is an endogenous agonist which binds better than glycine itself to the NMDA receptor (Panatier et al., 2006; Shleper et al., 2005). D-serine is synthetized by astrocytes from L-serine thanks to the enzyme serine racemase (SR), expressed mainly in glial cells and upregulated by glial activation (Wu and Barger, 2004). Overactivation of SR increases the production and the efflux of D-serine leading to a massive production of mitochondrial ROS and consequently overactivation of NMDA receptors. The increase in extracellular glutamate levels is considered one of the main causes of neuronal death (Van Damme et al., 2005; Bruijn et al., 2004). According to these evidences, we treated IMA 2.1 and N9 cell lines with increasing concentrations of L-serine [0, 10, 50 and 100 uM] in serum free DMEM for 24 hours. Equal volumes of medium (500µl) were collected and α-synuclein release was determined by western blot analysis. As shown in Figure 19 A, B the treatment with L- serine did not influence α-synuclein release in both the cell lines.
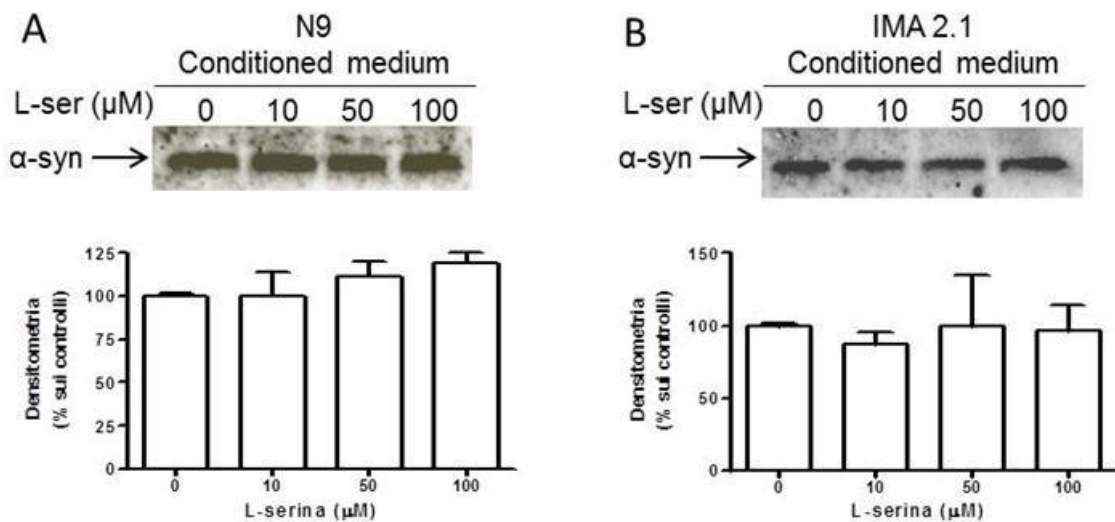
Fig.19. Effect of L-serine treatment on α-synuclein release.

Representative western blot analysis and densitometry of α-synuclein release from N9 (A) and IMA2.1 cells (B) stimulated for 24 hours with increasing L-serine concentrations [0; 10; 50; 100 µM]. Equal volumes (500 µl) of 24h-conditioned medium from microglia (A) and astrocytes (B) were concentrated and loaded. Each bar is the mean ± SE of 3 independent experiments compared to control. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.3.7 INTRACELLULAR EXPRESSION AND EXTRACELLULAR RELEASE OF α-SYNUCLEIN FROM N9 AND IMA 2.1 CELL LINES OVEREXPRESSING WT AND MUTANT α-SYNUCLEIN

Previous studies have shown that the A53T and A30P point mutations in the gene encoding for α-synuclein are related to the onset of an autosomal dominant juvenile form of Parkinson's disease (PD) (Singleton et al., 2013), while multiplication of the entire locus seems to lead to an earlier and more severe onset of the disease depending on the number of gene copies (Fuchs et al., 2007; Farrer et al., 2004; Singleton et al ., 2003). According to the literature and to our data on the release of mutant SOD1 from primary glial cultures, it has been investigated whether overexpressed wild-type α-synuclein, or mutant A53T α-synuclein and A30P α-synuclein, could show the same secretion alterations observed for SOD1. We transiently overexpressed wild-type synuclein and familial PD-linked α-synuclein mutants (A53T and A30P, kind gift from Dott. Nelson Cole, Department of Health & Human Services. National Institute of Health, Maryland) on N9 and IMA2.1 cell lines. Western blot analysis performed on lysed cells 24 hours after transfection, showed no difference in the intracellular expression of α-synuclein between the wild-type and mutant forms overexpressed in the N9 cell line (Fig.18A), though an evident intracellular accumulation of mutant α-synuclein, with an even more evident accumulation of A53T α-synuclein, compared to wild-type α-synuclein was observed in IMA2.1 cells (Fig.20B). Western blot analysis performed on conditioned media 24 h after transfection with

A53T α-synuclein and A30P α-synuclein plasmids showed that cells overexpressing mutant α-synuclein release more protein than wild-type α-synuclein overexpressing cells in the conditioned medium (Fog.20C, D) However, both cells lines seemed to respond in different ways to α-synuclein mutations; microglial cells expressed a similar amount of A53T and A30P α-synuclein (Fig.20A), but showed an increase in mutant α-synuclein release, being more evident for A53T α-synuclein (Fig. 20C). IMA 2.1 cells showed an increased expression of both mutants of α-synuclein than wild-type α-synuclein, this increase was much more evident for A53T α-synuclein (Fig. 20B), yet IMA 2.1 cells seem to balance the different mutant α-synuclein expression through secretion (Fig. 20D) since higher secretion levels of A30P α-synuclein than A53T α-synuclein were observed.
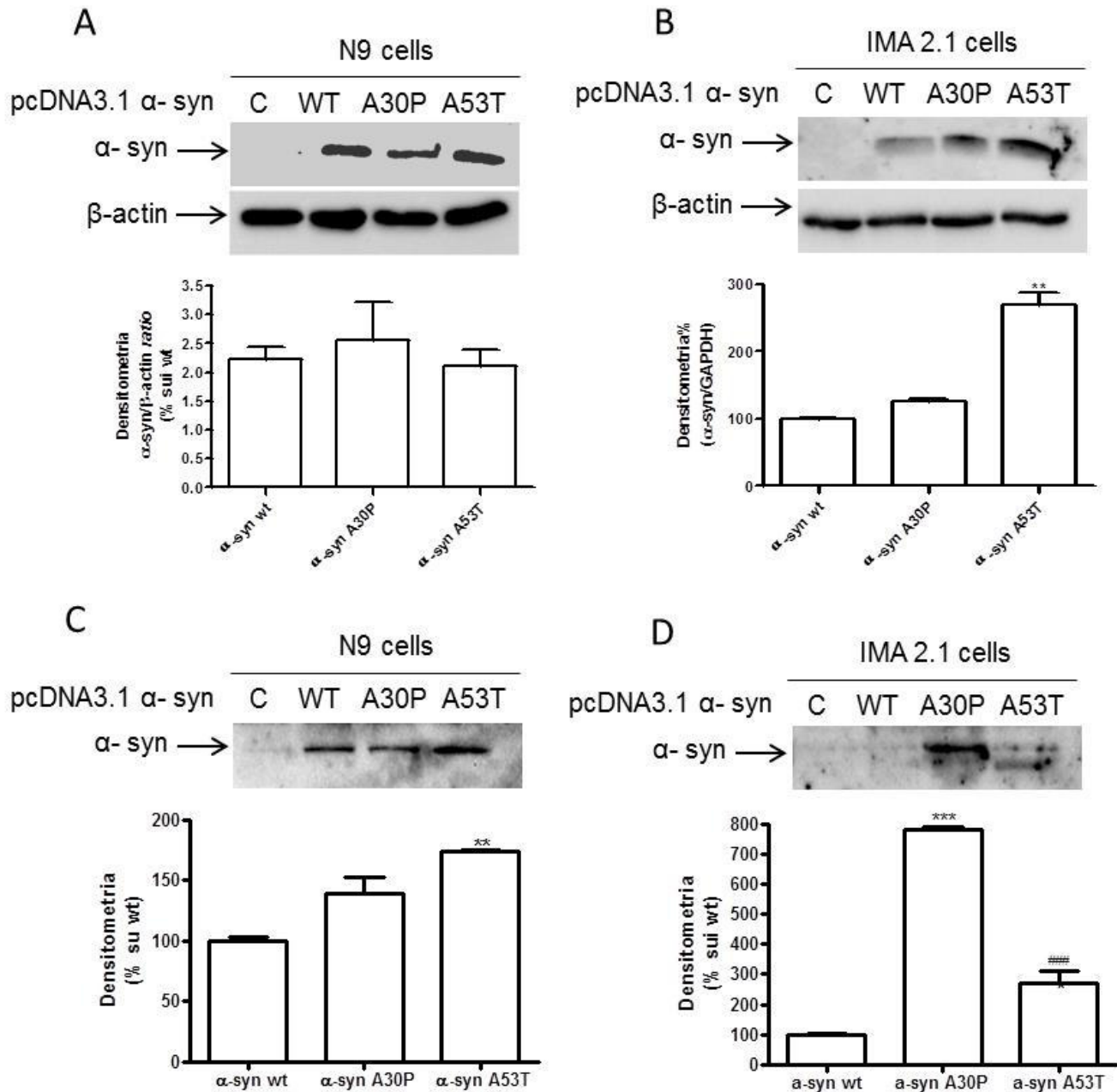
Fig.20. Altered α-synuclein release in microglia and astrocytes overexpressing mutant α-synuclein.

Western blot analysis and relative densitometry of α-synuclein expressed (A, B) and released (C, D) in 24h conditioned medium by microglia (A, C) and astrocytes (B, D) overexpressing wild-type α-synuclein, A53T and A30P α-synuclein. Each bar represents the mean ± SE of 3 different experiments normalized to β-actin. Equal volumes (500 μl) of 24h-conditioned media were concentrated and loaded in parallel. Each bar represents the mean ± SE of 3 different experiments normalized to the control. ** p < 0.01;

***p<0.001 compared to the control; ### p < 0.001 compared to A53T α-synuclein. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.3.8 EFFECT OF WILD-TYPE AND MUTANT α-SYNUCLEIN OVEREXPRESSION ON MICROGLIA AND ASTROCYTE ACTIVATION

Protein intracellular accumulation, observed in many proteinopathies, seems to be responsible for glial cell activation (Ferretti et al., 2012). Increase in the intracellular expression of iNOS and the consequent release of NO is considered a marker of glial activation (Yuste et al., 2015). In microglial cells, wild-type α-synuclein overexpression slightly increased iNOS expression, as shown by Western blot analysis (Fig.21A), while nitrite levels in the medium (Fig.21C) did not show significant differences between control and overexpression. Overexpression of mutant and wild-type α -synuclein in astrocytes did not cause any difference in iNOS expression (Fig.21B) and nitrite levels (Fig.21D), further underling that α-synuclein overexpression does not alter glial activation.
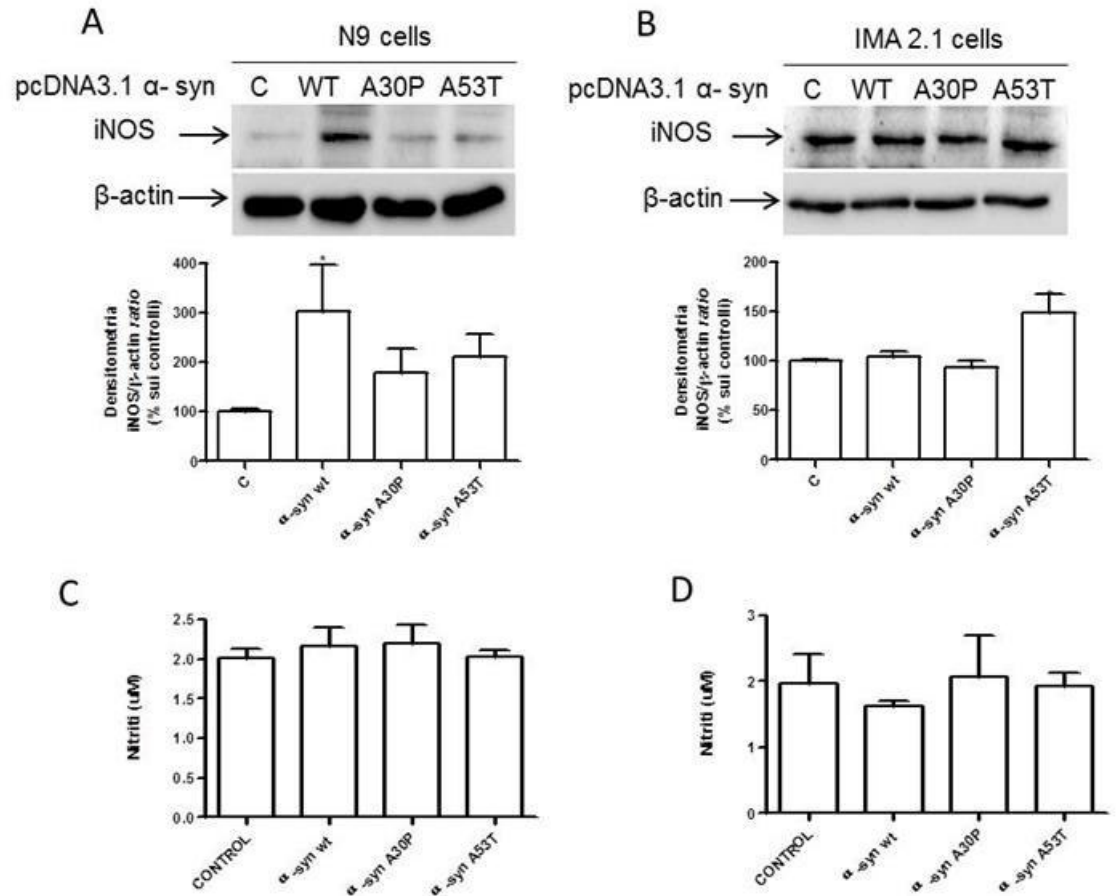
Fig.21. Effect of wild-type and mutant α-synuclein overexpression on microglia and astrocyte activation.

Western blot and densitometry of N9 cells (A) and IMA 2.1 cells (B) show a partial activation of N9 cells due to wild-type α-synuclein overexpression (A) but no evident differencesin IMA 2.1 cell activation (B), while the A53T mutation induces the activation IMA 2.1 cells. In addition, there were no significant differences in nitrite release from N9 (C) and IMA2.1 cells (D). Each bar represents the mean ± SE of 3 different experiments normalized to β-actin. * $p < 0.05$, compared to the control. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

4.3.9 EFFECT OF WILD-TYPE AND MUTANT α-SYNUCLEIN OVEREXPRESSION ON MICROGLIA AND ASTROCYTE VIABILITY

Cells survival/death was evaluated by counting condensed nuclei, considered an index of apoptosis, and total nuclei, after Hoechst staining.

N9 cells (Fig. 22A) and IMA2.1 cells (Fig.22B) overexpressing wild-type α-synuclein, A53T type α-synuclein and A30P type α-synuclein were fixed in 4% PFA 24 hours after transfection and nuclei stained with Hoechst. As shown in Fig. 20C related to N9 cells and Fig.20D to IMA 2.1 cells, nuclei counting revealed an increase in apoptosis in both cell lines overexpressing wild-type and mutatnt α-synuclein. Microglia (Fig. 22C) seem to be more affected by A53T α-synuclein than by A30P type α-synuclein, however overexpression of wild-type α-synuclein also promoted apoptosis. Astrocytes (Fig. 22D) seem to be more affected by A53T α-synuclein than by A30P α-synuclein, the same mutation previously showed to increase iNOS expression in IMA2.1 cells.
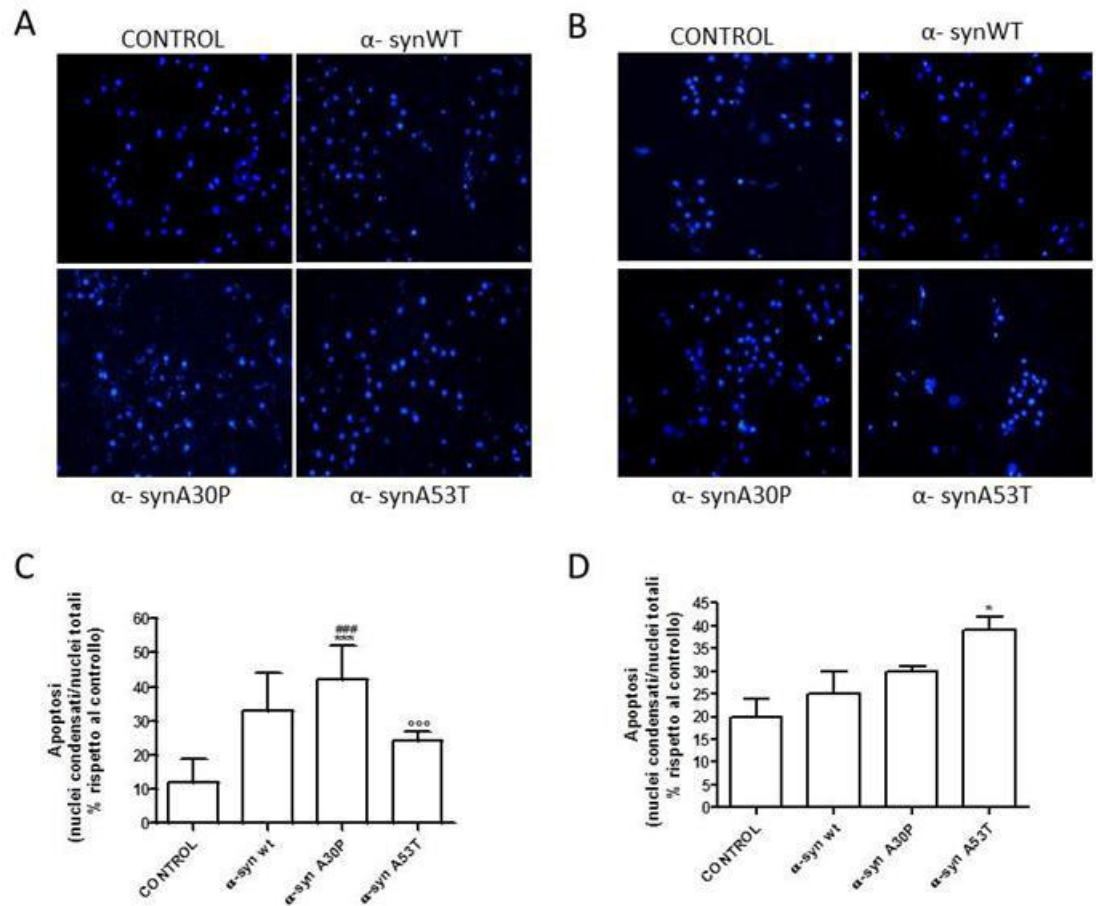
Fig.22. Effect α-synuclein overexpression on glial cell survival/death.

Hoechst staining (A, B) and nuclei counting (C, D) of condensed/total nuclei from microglia (A) or astrocytes (B) overexpressing wild-type or mutant α-synuclein. Each bar is the mean ±S.E. of three independent experiments expressed as the ratio between condensed and total nuclei. * p < 0.05, *** p <0.001 relative to control; ### p < 0.001 relative to A53T α-synuclein. Bonferroni's post-hoc test following one-way ANOVA.

4.3.10 GLUTAMATE AND SERINE RELEASE BY MICROGLIA AND ASTOCYTES OVEREXPRESSING WT AND MUTANT α-SYNUCLEIN

Concerning the role of glutamate in glutamate excitotoxicity (Van Damme et al., 2005; Bruijn et al., 2004) and the role of glial cells in the production of serine (Bezzi et al., 2004; Schell et al., 1995), we decided to

measure the levels of glutamate and serine through HPLC in 24 h conditioned media from microglia and astrocytes overexpressing wild-type α-synuclein, A53T α-synuclein and A30P α-synuclein. As shown in table 3, 24 h conditioned medium from N9 cells contains higher amounts of glutamate compared to serine. On the contrary, in 24 h conditioned medium from IMA 2.1 cells, lower levels of glutamate and a greater amounts of serine were observed. However, in conditioned media from both glial cells, no significant differences were observed following wild-type α-synuclein, A53T α-synuclein and A30P α-synuclein overexpression, thus indicating that the presence of mutant α-synuclein did not interfere with the release of these two important aminoacids, at least in our experimental conditions.

	N9		IMA 2.1	
	Glu (µM)	Ser (µM)	Glu (µM)	Ser (µM)
Controllo	171,3±16,1	16,0±1,3	52,5±12,7	171,8±1,8
A-syn wt	161,3±17,9	29,3±6,4	24,5±7,8	182,5±3,7
A-syn A30P	162,7±17,7	21,7±3,0	27,0±9,8	192,5±1,2
A-syn A53T	165,7±18,5	31,3±9,9	42,0±8,2	180,0±8,2

Table 3. HPLC measurement of glutamate and serine levels in 24 h conditioned media.

HPLC measurement of glutamate and serine levels in 24h conditioned media from microglia and astrocytes overexpressing wild-type, A53T α-synuclein and A30P α-synuclein.The table quantifies the µM concentration of each amino acid and each value is the average of three / four experiments ± SE. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test.

5. DISCUSSION

In this thesis, I presented data on the release of three different proteins; PNP, SOD1 and α-synuclein, by microglial cells. The main interest on these proteins derived from the fact that they are all involved in brain neuropathology, as mutations in the gene codifying for PNP induce PNP deficiency, a neurodevelopmental disorder characterized by immunodeficiency, while SOD1 and α-synuclein are both involved in neurodegenerative diseases, amyotrophic lateral sclerosis and Parkison's disease respectively, both characterized by the presence of protein aggregates, neurodegeneration and neuroinflammation. Therefore, in all these neurological diseases there is a strong involvement of immune cells, however whether and how microglia, the brain immune cells, could be related has not been thoroughly studied. In particular, considering that previous results from our laboratory clearly demonstrated that microglial cells exert a neuroprotective role through the release of soluble factors (Polazzi et al., 2001, 2009, 2013, 2015; Polazzi & Monti, 2010) and that SOD1 is one of them, in this thesis we decided i) to check whether other proteins related to neurological diseases were released, ii) to study whether microglial cells could also release factors though exosomes and iii) to study the effect of different physiopathological stimuli on microglial protein release. We performed these studies by using two main in vitro models: i) primary cultures of glial cells and ii) glial cell lines. In vitro models allow using biochemical and molecular approaches in a "clean" cell system with no contamination by other brain cells, a crucial issue to clearly understand such complex biological processes. Primary cultures are widely considered the more physiological models to study glial cells, but they present bioethical issues related to the use of animals for experimental purposes. Therefore, for the last part of my thesis, we used glial cell lines, IMA2.1 astrocytes and N9 microglia, which are both immortalized mouse primary glial cells.

5.1 PNP RELEASE BY PRIMARY GLIAL CELLS

The study of PNP expression in the brain started in the early '90s, when it was demonstrated that in brain slices PNP co-localized with GFAP, an astrocytic marker, suggesting that PNP is primarily expressed by glial cells and not by neurons (Castellano et al., 1990). More recently, PNP expression has been reported mainly in microglial cells, astrocytes, blood vessels and in some nerve fibers, suggesting that glial cells are crucial for maintaining cerebral extracellular purine levels. Thus, the aim of this study was to demonstrate a cell-specific PNP expression in the CNS. Using rat primary *in vitro* neuronal, astrocyte and microglia cultures (Bilimoria, Bonni, 2008; Contestabile, 2002; De Vellis, Cole, 2012; Hertz et al., 1998), we confirmed that PNP is expressed by glial cells, both at the mRNA and protein levels, to a greater extent compared to neurons and that PNP is also constitutively released by these cells. The presence of considerable amounts of PNP in basal conditions allowed us to hypothesize that this enzyme is involved in physiological functions in the central nervous system (CNS). Indeed, it is known that glial cells cooperate with neurons in fundamental processes, such as neural plasticity and neurogenesis, as well as learning and memory, mainly through the release of soluble molecules, including purines (Bitzer-Quintero, González-Burgos, 2012). The role played by microglial cells and astrocytes in the brain immune response is also well known. Actually, microglia and astrocytes exert their immune function mainly in the inflammatory process following acute and chronic neurodegenerative diseases, among which psychiatric disorders are included. In these pathological conditions, glial cells become activated (Eikelenboom et al., 2012) and exert their function by releasing several factors. For this reason, we decided to study whether PNP release from neural cells could be altered by glial cell activation after neuroinflammatory stimuli. We used bacterial LPS and IFN-γ, which are able to induce a wide range of inflammatory activities: increased phagocytosis, chemotaxis, cytokine secretion, activation of the respiratory burst and induction of nitric oxide synthase (Zielasek, Hartung, 1996). Additionally, we used ATP, which is released by injured and dying cells leading to activation of P2X7 receptors in

glial cells (Franke et al., 2006). Neither LPS nor IFN-γ modified PNP release by any brain cell type, clearly indicating that these two inflammatory agents, usually able to activate glial cells in pathological conditions are not involved in the modulation of PNP release. On the contrary, we showed for the first time that ATP increases PNP release from both astrocytes and microglia through activation of the P2X7 receptor. Indeed, Bz-ATP, a P2X7 rceptor agonist, induced PNP release, while the release of PNP was blocked by the specific P2X7 receptor antagonist Ox-ATP (Michel et al., 2000) but not by the non-specific P2Y receptor antagonist suramin, underling the central role of P2X7 receptors in PNP release. Since ATP is released into the extracellular space either by exocytosis or by damaged and dying cells, this could be reasonably considered as one of the first signals used by neurons to activate glial cells in order to increase their neuroprotective potential stimulating the release of neuroprotective molecules from glial cells (Skaper et al., 2011).

It is known that cell communication is mediated not only by molecules freely released in the extracellular space, but also through vesicular traffic and in most neurodegenerative diseases, protein release may be altered. Here, we showed that PNP release is mediated by vesicles secreted by glial cells especially following ATP stimulation. Interestingly, our data are also in agreement with findings indicating that this non-classical secretory pathway is modulated by ATP through P2X7 receptors in brain immune cells (Dubyak et al., 2012). In fact, in presence of exogenous ATP, there was an increased co-localization of PNP with the lysosomal protein LAMP-1 in microglia and astrocytes. Moreover, ATP also enhanced the amount of PNP and LAMP-1 contained in secreted EVs, which potentially transport proteins, enzymes and RNAs from one cell to another and are key players in intercellular signaling with an emerging role of released vesicles in neuron-glia communication and as support to other glial cells and neurons (Frühbeis et al., 2013). PNP deficiency mainly affects the immune system, astrocytes and especially microglia, the immune cells of the brain, implicated in modulating this system. In fact, in pathological conditions, both cells show a continuous cross-talk with CNS-infiltrating T-cells and other components of the innate immune system, mainly by releasing several molecules (Ransohoff. et al., 2012). Thus, in this

framework, our study on PNP expression, sub-cellular localization, secretion and release regulation in glial cells opens the way to clarify the physiological role of this enzyme and casts light on the pathological mechanisms underlying PNP-deficiency.

5.2 SOD1 RELEASE BY PRIMARY GLIAL CELLS IN PHYSIOPATHOLOGICAL CONDITIONS

Following the previous results on PNP release from glial cells and previous studies from our laboratory on the neuroprotective role of microglial cells against neuronal damage (Polazzi et al., 2013), the second part of the project focused on the role that proteins secreted from glial cells may have in the onset and progression of some of the well-known neurodegenerative diseases.

Polazzi et al., demonstrated a neuroprotective role of microglia exerted by low molecular weight secreted proteins. SOD1 was identified as one of these molecules, constitutively produced, released and accumulated in conditioned medium (MCM). On one hand, SOD1 seems to be a promising molecule, together with other low molecular weight secreted proteins, to restore injured neurons; on the other hand, misfolding, intracellular aggregation and mutations in SOD1 are some of the main hallmarks observed in patients affected by familial Amyotrophic Latera Sclerosis (ALS). Glial cells appeared to be crucial for the initiation and progression of the disease and a non-cell autonomous mechanism for ALS neurodegeneration has been postulated (Ilieva et al, 2009). Mutant SOD1 has been shown to cause a reduction in glial cell physiological function, activation of microglial cells characterized by an elevated production of cytotoxic molecules such as ROS, inflammatory molecules including iNOS, pro-inflammatory cytokines and failure of astrocyte glutamate uptake, thus suggesting that motor neuron degeneration could be triggered by glial cell dysfunction (Weydt et al., 2004; Hensley et al., 2006; Liao et al., 2012; Frakes et al., 2014). However, whether neurodegeneration is linked to a loss of physiological function or to a gain of toxic action by glial cells and whether astrocytes and microglial cells have the same behavior in

ALS is not known. Since 20% of familial ALS are caused by mutations in the SOD1 gene and SOD1 alterations are also involved in sporadic ALS (Rosen et al., 1993), here we studied the release of wild-type SOD1 and SOD1 mutations linked to ALS. To this aim, we overexpressed in rat primary cultures of microglia and astrocytes wild-type and mutant SOD1 (two most-common mutations linked to ALS, G93A and A4V), to compare the effect of different mutations and we observed a reduction in the release of mutant SOD1 compared to wild-type SOD1 in both types of glial cells. It has been shown that SOD1 is released through exosomes by mouse motor neuron-like NSC-34 cells and astrocytes in ALS cell models (Gomes et al., 2007; Basso et al., 2013), leading to propose a prion-like mechanism for ALS pathology mediated by mutant SOD1. Here we showed that also microglial cells release SOD1 through vesicles.

For the first time we showed a reduction in SOD1 release, independently from its release mechanisms with parallel intracellular accumulation in both microglia and astrocytes. On one hand, this could indicate a reduction in neuroprotection by glial cells, as released SOD1 has been demonstrated to exert a neuroprotective effect (Polazzi et al., 2013); on the other hand, the intracellular accumulation of non-released SOD1 could alter the properties of glial cells possibly leading to a physiological dysfunction or to the activation of these cells. In fact, neuroinflammation and glial cells activation have demonstrated to be a characteristic hallmark of most neurodegenerative diseases, including ALS (Robberecht, 2011). Furthermore, the intracellular co-localization of accumulated mutant SOD1 and LAMP-1 seems to indicate a lysosomal dysfunction that could be the linked to decreased protein release by glial cells and to autophagy alterations. Growing evidence implicates autophagy defects as a potential cause of ALS pathology as well as in many other neurodegenerative diseases (Lee et al., 2015; Castillo et al., 2013).

Dysfunction in autophagic degradation pathways has been found in sALS and fALS patients, as well as in transgenic mouse models of SOD1-fALS (Li et al., 2015), but all data focus on neurons. Here we showed that a notable reduction in microtubule-associated protein 1 light chain 3 (LC3 II)

expression in microglial cells overexpressing mutant SOD1 compared to wild-type SOD1. LC3 is a cytosolic protein, which is lipidated to LC3II during the formation of autophagosomes, and localizes on autophagosomes and autolysosomes. LC3-II levels are considered a good indicator of autophagosome formation and therefore autophagy (Tanida et al., 2004). We could assume that the intracellular accumulation of G93A SOD1 and A4V SOD1 in microglial cells may be linked to a blockade of autophagy, even if we cannot speculate whether the presence of mutant SOD1 causes the intracellular accumulation of these proteins and therefore the autophagy impairment or, on the contrary, whether mutant SOD1 blocks autophagy determining the consequent intracellular accumulation of SOD1. Interestingly, this happens in microglial cells only and not in astrocytes, while SOD1 accumulates in both types of glial cells. In particular, LC3II expression is undetectable in primary astrocytes, but we could consider that these cells have a very low metabolic rate. In parallel, we detected a decrease in the ratio between phosphorylated and total mTOR in microglial cells. This was apparently in contrasts with LC3II expression, but it may also indicate an mTOR-independent autophagy pathway, as in other neurodegenerative diseases, like Huntington and Parkinson's disease (Sarkar et al., 2007), or microglial activation and neuroinflammation (Lisi et al., 2014). In agreement with the data on autophagic markers, here we demonstrate that overexpression of mutant SOD1 determines the activation of microglial cells, but not of astrocytes, as indicated by increased iNOS expression and nitrite production. Microglial cells are the resident immune cells of the CNS. In physiological conditions, "resting" microglia supervise and maintain homeostasis of the brain environment. In response to pathological insults, microglia become activated. The first microglial activation is beneficial for damage repair, but prolonged activation produces chronic inflammation, which leads to the release of reactive oxygen and nitrite species, pro-inflammarory cytokines and the loss of physiological neuronal support. Chronic activation of microglia is often accompanied by their phenotypic shift from the neuroprotective M2 (alternatively-activated) to the toxic M1 (classically-activated) state (Zhao et al., 2013). Here, we showed that microglial cells overexpressing mutant SOD1 are activated, but they

preserve their M2 state, as they continue to express two M2 markers, the Triggering Receptor Expressed on Myeloid Cell 2 (TREM2) and the Mannose Receptor C Type1 (MRC1). Indeed, the functional states of microglia during the progression of ALS have not been fully characterized, but *in vivo* studies on ALS SOD1 mice indicate that there is a transformation of microglia expressing mutant SOD1 from a neuroprotective M2 phenotype at disease onset to a cytotoxic M1 phenotype during disease progression (Liao et al., 2012). Actually, we cannot specify the stage of ALS disease we are representing by *in vitro* SOD1 overexpression on primary glial cell cultures, however, considering the results, we can speculate that our cellular model of ALS mimics an early/middle stage of the disease.

Furthermore, the neuroprotective role of microglial conditioned medium against neuronal damage has been demonstrated (Polazzi et al., 2013) being SOD1 one of the neuroprotective molecules released by microglial cells (Eleuteri et al., 2008). Therefore, here we tested the neuroprotective effect of glial cells overexpressing wild-type or mutant SOD1 on cerebellar granule neurons, a widely-used and accepted model for neurodegeneration/neuroprotection studies, sensitive to glutamate excitotoxicity (Contestabile, 2002). We observed that microglia exert a neuroprotective effect independently from the type of SOD1 overexpressed, while overexpression of mutant SOD1 by microglial cells appears to be toxic for neurons in control conditions. On the other hand, astrocytes do not appear to be neuroprotective by themselves, but neither to increase neuronal damage due to glutamate excitotoxicity. However, astrocytes become slightly neuroprotective when they overexpress wild-type SOD1. Taken together, these data seem to indicate that microglia and astrocytes play different roles in neuroprotection/neurodegeneration, depending on the type of SOD1 expressed (wild-type vs. mutant) and on the physiopathological environment of neurons (control vs. neurotoxic conditions), while the literature tends to include both types of glial cells in the same molecular mechanisms (Puentes et al., 2016). Significant evidence links glutamate and serine release to motor neuron death in ALS non-cell autonomous neurodegeneration, as D-serine is a co-agonist of the N-methyl-D-Aspartate receptor, and serine racemase (SR) is mainly

expressed in glial cells and upregulated by glial activation, as well as in spinal cord of ALS patient and in SOD1 ALS mouse models (Paul, De Belleroche, 2014). However, here we measured glutamate and serine in 24 h conditioned media from primary rat microglia and astrocytes overexpressing wild-type or mutant SOD1, but we did not observe any significant difference. Therefore the neurodegenerative/neuroprotective effect of glial cells in co-culture seems not to be related to changes in the release of these aminoacids.

Trehalose has been proposed to promote the clearance of autophagy substrates such as mutant huntingtin protein or the A30P and A53T mutants of α-synuclein (Sarkar S. et al., 2007). Oral administration of trehalose in a transgenic mouse model of HD decreased polyglutamine aggregates in the brain and liver, improved motor function and extended lifespan (Tanaka M. et al., 2004). It has also been reported that trehalose treatment can ameliorate behavioral and pathological symptoms in a mouse model of tauopathy (Rodriguez-Navarro J.A. et al., 2010). Additionally, a recent study on the G93A SOD1 mouse model showed how daily administration of 2% (w/v) trehalose significantly reduced the levels of mutant SOD1 and p62 and increased LC3-II levels in the spinal cord of 90-day-old G93A SOD1 transgenic mice. Furthermore, independently of autophagy, trehalose consistently inhibited microgliosis and astrogliosis throughout the entire duration of the study (Li et al., 2015).

In this study, we showed, for the first time, the role of trehalose in reducing intracellular accumulation of mutant SOD1 in glial cells and consequently the reduction of the activate state of microglia. Consistent with the previous data on the impairment of authophagy in microglial cells overexpressing mutant SOD1, trehalose treatment significantly reduced this pathological state allowing us to speculate on the promising use of this disaccharide in restoring the autophagic flux promoting the initiation of autophagy and increased lysosomal biogenesis to reduce microglial activation and neuroinflammation. We mentioned the neuroprotective role of microglia in case of neuronal damage (glutamate excitotoxicity), but, at the same time, overexpression of mutant SOD1 on microglial cells appeared to be toxic by themselves for neurons in control conditions. Co-treatment of co-culture with

trehalose and glutamate showed a great ability of trehalose not only to reduce glutamate excitotoxicity in neurons, but rather much more interesting, to reduce the toxicity of microglial cells overexpressing G93A SOD1 and A4V SOD1, restoring neuronal viability even in control conditions. Taken together, these data suggest an involvement of autophagy impairment in glial cells in the accumulation of proteins forming aggregates in proteinopathies such as ALS, and therefore in the onset and progression of neurodegenerative diseases, thus showing how restoring the physiological functions of glia through autophagy induction could be an innovative way to reduce neuronal death.

5.3 ALPHA-SYNUCLEIN RELEASE BY GLIAL CELL LINES

Similarly to our studies on the role of SOD1 in glial cell pathophysiology in ALS, we carried on a parallel study on α-synuclein expression and release by glial cells as well as on the effect of wild-type or familiar PD-linked mutant α-synuclein overxpression. Although the physiological function of α-synuclein has not yet been clarified, its interaction with synaptic vesicles has been shown; α-synuclein is generally associated to the pre-synaptic membrane, is involved in the maintenance of membrane integrity during the release of neurotransmitters (Eliezer et al ., 2001) and in vesicle trafficking and fusion during signal transmission at the synaptic level (Auluck et al., 2010; Iwai. et al., 1995). It is also known that α-synuclein is transmitted from neuron to neuron and astrocyte through endocytosis and inclusion bodies (Lee et al., 2010; Rappold, Tieu, 2010), but whether it can also released by microglial cells has not been yet clarified. So far, most studies have mainly investigated the role of α-synuclein in neurons, excluding non-neuronal cells such as glial cells. Parkinson's disease (PD), however, involves the alteration of many other cell types in addition to neurons, modifying not only neuronal function, but also the cross-talk between different neurons and neighbouring cells (Appel et al., 2010). According to the "non-cell autonomous" hypothesis, which assesses the role of glial cells in

neurodegenerative diseases, and considering the previous results on glial cell release of molecules even through vesicles, we started a preliminary study on the expression and release of α-synuclein in physiopathologycal conditions. α-synuclein is transmitted from neuron to neuron and from neuron to astrocyte through endocytosis and inclusion bodies (Rappold et al., 2010; Lee et al., 2010), but it is still unclear whether it is even released by microglial cells. We demonstrated for the first time that α-synuclein is expressed not only in neurons but also in microglial cells, both at the mRNA and protein levels. At the same time, we demonstrated α-synuclein release, through vesicles, not only by neurons as showed in the literature, but also by microglia and astrocytes in physiological conditions. The "spot" localization of α-synuclein and the partial co-localization with LAMP-1 allowed us to speculate on a localization in secretory granules, probably of lysosomal nature (Takenouchi et al., 2009).

Neurodegenerative diseases, stroke, trauma and hypoxia, affect neuronal survival indirectly by triggering the state of neuroinflammation. In these conditions, microglia is activated in response to insults adopting a phagocytic phenotype and releasing pro-inflammatory mediators, cytokines and chemokines. The acute neuroinflammatory response seems to be beneficial to the CNS, as it tries to reduce the injury and restore damaged tissues (Wake. et al., 2013, Kiefer et al., 1995, Streit et al., 1999, Imai et al., 1997, Lalancette-He´ bert et al., 2007, Yanagisawa et al., 2008, Madinier et al., 2009). On the other hand, chronic neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), tauopathies and multiple sclerosis (MS), are known to be associated with chronic neuroinflammation. Classically activated microglia, mimed *in vitro* by LPS treatment, have pro-inflammatory, neurotoxic properties, inhibiting the proliferation of lymphocytes; M1 activated macrophages secrete pro-inflammatory cytokines such as interleukin IL-1α, IL-1β, tumor necrosis factors (TNF) and nitric oxide (NO). Activation of glial cells mediated by LPS treatment, was confirmed by the expression of inducible nitric oxide synthase (iNOS) and the release of nitrite in the medium. Classically activated microglia promote the release of α-synuclein. On one hand, this may be a defence mechanism necessary to preserve physiological functions because α-synuclein

accumulation and aggregation into oligomers or fibrils has a detrimental effect on cells (Irwin et al., 2013; Vekrellis et al., 2004). On the other hand, activation of glial cells may be a consequence and not the cause of neuronal damage; in this case, we could suppose that the increased release of α-synuclein occurs as a secondary event and could even increase damage also through vesicles, known to promote the trafficking of aggregated, damaged or misfolded proteins. On the contrary, ATP does not interfere with α-synuclein release in astrocytes and it partially increases the release by microglia. ATP activation of glial cells is less aggressive than LPS activation (Takato et al., 2009); the stimulus may be not strong enough to induce a significant inflammatory response in glial cells. Furthermore, to confirm this hypothesis, only the highest dose of ATP used was shown to activate microglial cells, while astrocytes seems to be more sensitive to this stimulus. Taken together, these results allowed us to speculate on the different role of glial cells in response to different stimuli.

The role of D-serine in the modulation of N-methyl-D-aspartate receptors (NMDAR) is knwn; it is synthetized by astrocytes from L-serine thanks to the enzyme serine racemase (SR), expressed mainly in glial cells and upregulated by glial activation (Wu and Barger, 2004). Glial cell activation stimulates the release of cell death mediators, such as glutamate, cytokines and reactive oxygen species (ROS) and the super-activation of SR enzyme that increases the production and efflux of D-serine (Barger and Wu, 2004) leading to over activation of NMDA receptors. The increase in extracellular glutamate levels is considered to be one of the main causes of neuronal death (Van Damme et al., 2005; Bruijn et al., 2004). Here, we show that L-serine does not modify α-synuclein release from any cell type, clearly indicating that the glutamate receptor is not involved in the pathway mediating α-synuclein release. We have demonstrated constitutive α-synuclein production and release by glia and the consequent alteration of the release in case of strong activation.

α-synuclein is also involved in neurodegenerative diseases; its pathogenicity is related to its propensity to aggregate as reported to be the main component of amyloid plaques in Alzheimer's patients (Campion et al. 1995).

α-synuclein fibrils are the main component of Lewy bodies in PD patients, but they are also found in other types of aggregates, Lewy neurites or oligodendroglial inclusions that characterize diseases indicated as "synucleinopathies" (Steiner et al., 2011; Kahle, 2008;). Missense mutations in the gene (A53T, A30P), or duplication or triplication of the wild-type form of α-synuclein are neurotoxic and responsible for familial forms of PD (Mosharov et al., 2006). Overexpression of human wild-type, A53T and A30P α-synuclein pushes microglial cells towards the reactive phenotype characterized by an increase in arachidonic acid metabolism, cytokine and interleukin secretion, reactive oxygen species, tumor necrosis factor α (TNF-α) and nitric oxide (NO) production (Rojanathammanee L. et al. 2011). According to the "non-cell autonomous" hypothesis and considering our previous results on α-synuclein release by glial cells, we decided to focus on the release of wild-type and mutant α-synuclein. To this aim, we overexpressed in immortalized cell lines of microglia and astrocytes wild-type and familial PD-linked mutant α-synuclein (A53T and A30P), to compare the effect of different mutations. We observed an increase of mutant α-synuclein release compared to wild-type α-synuclein in both types of glial cells but with differences between both mutations and both cell types. This was not surprising, as it has been previously demonstrated that the amount of α-synuclein released after LPS treatment is increased, leading to propose a prion-like mechanism. We could also hypothesize that overexpression wild-type α-synuclein was able to determine the activation of microglial cells, but not of astrocytes, as indicated by increased iNOS expression. We cannot specify the disease stage we are representing by *in vitro* α-synuclein overexpression but considering the results, we can speculate that our cellular model mimics an early stage of the disease. Overexpression of α-synuclein, even only wild-type α-synuclein, promotes apoptosis in glial cells, being more evident following mutant α-synuclein overexpression. Taken together, these data may suggest that in case of strong damage, LPS treatment *in vitro* or Parkinson's Disease mimed by overexpression of wild-type or mutant α-synuclein (A53T and A30P), activated glial cells release more α-synuclein. In accordance to the "non-cell

autonomous" mechanism and to the release of vesicles, α-synuclein released in these conditions may contribute to PD.

6. CONCLUSIONS

In this thesis, we performed parallel studies on glial cell models regarding in vitro release of three different proteins, Purine Nucleoside Phosphorylase (PNP), Superoxide dismutase-1 (SOD1) and α-synuclein (α-syn), which are all linked to neuropathological conditions. Our findings demonstrate that astrocytes and microglia, not only express but also release PNP, SOD1 and α-synuclein, even through vesicles, and that this release is modulated in different neuropathological conditions.

PNP is a key enzyme in extracellular metabolism and the salvage pathway of adenine- and guanine-based purines. This enzyme is altered in PNP-deficiency, a neurological condition characterized by neurodevelopmental delay and immunodeficiency, where glial cells could play an important neuroprotective role, though this has never been studied. Here, we obtained data on PNP release by glial cells and we could speculate that, by releasing this enzyme, glial cells may support neuronal activity, by maintaining homeostasis of the purinergic system. In particular, since cerebellar neurons displayed low levels and reduced ability to release PNP, we hypothesized that glial PNP is particularly important for neuronal function.

SOD1 is an enzyme crucial in both familial and sporadic ALS. Previous data from our laboratory and other groups have demonstrated that glial cells release SOD1, being involved in neuroprotection. Here, by using in vitro models of rat primary cultures of glial cells overexpressing wild-type or mutant SOD1, we observed that fALS-linked SOD1 mutants are less released than wild-type SOD1. In parallel, mutant SOD1 accumulates intracellularly and this can be linked to autophagy impairment, which in turn leads to glial activation. However, whether intracellular SOD1 accumulation is due to autophagy impairment or rather autophagy impairment could induce the intracellular accumulation of SOD1 remains unclear. Nonetheless, activation of microglial cells might be responsible for reduced neuroprotection against glutamate excitotoxicity in co-cultures with cerebellar granule neurons. Trehalose, which is able to induce autophagy, seems to restore the physiological expression and release of mutant SOD1, as well as the physiological neuroprotective role of

microglia. Astrocytes seem to be less susceptible to mutant SOD1 overexpression and do not display neuroprotective functions against glutamate excitotoxicity, while even overexpression of wild-type SOD1 seems to provide neuroprotection.

Finally, we focused on α-synuclein release by glial cells. While it is well-known that neurons and astrocytes release this protein, microglial cells have never been studied in this regard. By using immortalized mouse astrocyte and microglia cell lines, we observed that familial PD-linked α-synuclein mutants are more released than wild-type α-synuclein, in accordance to the increased release promoted by activated glial cells. The increased toxicity due to overexpression of mutant α-synuclein together with the increased release might be responsible for reduced neuroprotection and for the diffusion of potential toxic molecules which support the prion-like hypothesis for Parkinson's Disease.

Taken together, these data propose a crucial role of glial cells regarding protein release, at least in *in vitro* models, and suggest to deeply study this mechanism in order to find potential biomarkers for diagnosis and targets for the treatment of neurodegenerative diseases.

7 References

Al-Chalabi A, Leigh PN., (2005). "Trouble on the pitch: are professional football players at increased risk of developing amyotrophic lateral sclerosis" Brain 128:451-3.

Aloisi F., (2001). "Immune function of microglia". Glia 36:165–179.

Angulo MC, Kozlov AS, Charpak S, Audinat E (2004) "Glutamate released from glial cells synchronizes neuronal activity in the hippocampus". J Neurosci 24(31):6920–6927.

Appel SH, Beers DR, Henkel JS, (2009). "T cell-microglial dialogue in Parkinson's disease and amyotrophic lateral scle-rosis: are we listening?". Trends Immunol. 2010 Jan;31(1):7-17. doi: 10.1016/j.it.2009.09.003.

Arpaia E, Benveniste P, Di Cristofano A, Gu Y, Dalal I, Kelly S, Hershfield M, Pandolfi PP, Roifman CM, Cohen A, (2000). "Mitochondrial basis for immune deficiency. Evidence from purine nucleoside phosphorylase-deficient mice". J Exp Med., 191, 2197-208.

Auluck PK, Caraveo G, Lindquist S, (2010)."α-Synuclein: membrane interactions and toxicity in Parkinson's disease.", Annu Rev Cell Dev Biol;26:211-33.

Balakrishnan K, Verma D, O'Brien S, Kilpatrick JM, Chen Y, Tyler BF, Bickel S, Bantia S, Keating MJ, Kantarjian H, Gandhi V, Ravandi F, (2010). "Phase 2 and pharmacodynamic study of oral forodesine in patients with advanced, fludarabine-treated chronic lymphocytic leukemia". Blood. 116, 886-92.

Banati R.B., Gehrmann J., Schubert P., Kreutzberg G.W., (1993). "Cytotoxicity of microglia". Glia 7:111–118.

Bantia S, Kilpatrick JM, (2004). "Purine nucleoside phosphorylase inhibitors in T-cell malignancies". Curr Opin Drug Discov Devel., 7, 243-7.

Barres BA. 2008. "The mystery and magic of glia: A perspective on their roles in health and disease". Neuron 60:430–440.

Bartlett R, Stokes L, Sluyter R. (2014). "The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease". Pharmacol Rev., 66, 638-75.

Basso M, Pozzi S, Tortarolo M, Fiordaliso F, Bisighini C, Pasetto L, Spaltro G, Lidonnici D, Gensano F, Battaglia E, Bendotti C, Bonetto V. (2013). "Mutant copper-zinc superoxide dismutase induces protein secretion pathway alterations and exosome release in astrocytes: implications for disease spreading and motor neuron pathology in amyotrophic lateral sclerosis". J Biol Chem.;288(22):15699-711.

Battista D., Ferrari C.C., Gage F.H., Pitossi F.J. (2006). "Neurogenic Niche Modulation By Activated Microglia: Transforming Growth Factor Beta Increases Neurogenesis In The Adult Dentate Gyrus". Eur. J. Neurosci. 23, 83–93.

Beauvillain C., Donno S., Jarry U., Scotet M., Gascan H., Delneste Y., Guermonprez P., Jeannin P., Couez D., (2008). "Neonatal and adult microglia cross-present exogenous antigens". Glia 56:69–77.

Béchade Catherine, Cantaut-Belarif Yasmine and Bessis Alain. (2013). "Microglial Control Of Neuronal Activity". Front Cell Neurosci. 2013;7:32.

Bélanger M, Allaman I, Magistretti PJ, "Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation.", Cell Metab. 2011 Dec 7;14(6):724-38. doi: 10.1016/j.cmet.2011.08.016.

Bezzi P, Gundersen V, Galbete JL, Seifert G, Steinhäuser C, Pilati E, Volterra A, (2004). "Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate.", Nat Neurosci;7(6):613-20. Epub 2004 May 23.

Bianco F, Pravettoni E, Colombo A, Schenk U, Möller T, Matteoli M, Verderio C. (2005) "Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia". J Immunol., 174, 7268-77.

Bilimoria PM, Bonni A. (2008) "Cultures of cerebellar granule neurons". CSH Protoc., 13, 5107.

Bittner CX, Loaiza A, Ruminot I, Larenas V, Sotelo-Hitschfeld T, Gutiérrez R, Córdova A, Valdebenito R, Frommer WB, Barros LF, (2010). "High resolution measurement of the glycolytic rate.", Front Neuroenergetics.;2. pii: 26.

Bitzer-Quintero OK, González-Burgos I. (2012). "Immune system in the brain: a modulatory role on dendritic spine morphophysiology?" Neural Plast., 348642.

Boje KM, Arora PK, (1992)."Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death.", Brain Res ;587(2):250-6.

Bonvicini F., Marcello N., Mandrioli J., Pietrini V., Vinceti M., (2010). "Exposure to pesticides and risk of amyotrophic lateral sclerosis: a population-based case–control study". Ann. Ist. Super. Sanita 46 (3):284–287.

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP, (1995)."A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine.", Proc Natl Acad Sci U S A. ;92(16):7297-301.

Bové J, Perier C, (2012). "Neurotoxin-based models of Parkinson's disease.", Neuroscience.;211:51-76.

Bräuer AU, Nitsch R, Savaskan NE (2004). "Identification of macrophage/microglia activation factor (MAF) associated with late endosomes/lysosomes in microglial cells". FEBS Lett., 563, 41-8

Brown GC, Vilalta A. (2015). "How microglia kill neurons". Brain Res. 1628(Pt B):288-97.

Bruijn L.I., Miller T.M., Cleveland D.W.,(2004). "Unraveling the mechanisms involved in motor neuron degeneration in ALS". Annu. Rev. Neurosci. 27:723–749.

Bushong EA, Martone ME, Ellisman MH, (2004). "Maturation of astrocyte morphology and the establishment of as-trocyte domains during postnatal hippocampal development.", Int J Dev Neurosci. 22(2):73-86.

Bzowska A, Kulikowska E, Shugar D. (2000). "Purine nucleoside phosphorylases: properties, functions, and clinical aspects". Pharmacol Ther., 88, 349-425.

Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL, (2002). "Synaptic vesicle depletion correlates with attenuated synaptic responses to

prolonged repetitive stimulation in mice lacking α-synuclein.", J Neurosci.;22(20):8797-807.

Campion D, Martin C, Heilig R, Charbonnier F, Moreau V, Flaman JM, Petit JL, Hannequin D, Brice A, Frebourg T, (1995). "The NACP/synuclein gene: chromosomal assignment and screening for alterations in Alzheimer disease.", Genomics.;26(2):254-7.

Castellano B, González B, Finsen BR, Zimmer J. (1990) "Histochemical demonstration of purine nucleoside phosphorylase (PNPase) in microglial and astroglial cells of adult rat brain". J Histochem Cytochem., 38, 1535-9.

Castillo K, Nassif M, Valenzuela V, Rojas F, Matus S, Mercado G, Court FA, van Zundert B, Hetz C. (2013). "Trehalose delays the progression of amyotrophic lateral sclerosis by enhancing autophagy in motoneurons". Autophagy.;9(9):1308-20.

Chandra S, Fornai F, Kwon HB, Yazdani U, Atasoy D, Liu X, Hammer RE, Battaglia G, German DC, Castillo PE, Südhof TC, (2004). "Double-knockout mice for α- and beta-synucleins: effect on synaptic functions.", Proc Natl Acad Sci U S A.;101(41):14966-71.

Chen Q, Haddad GG (2004) "Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals". J Exp Biol 207:3125–3129.

Chinta SJ, Mallajosyula JK, Rane A, Andersen JK, (2010). "Mitochondrial α-synuclein accumulation impairs complex I function in dopaminergic neurons and results in increased mitophagy *in vivo*.", Neurosci Lett.;486(3):235-9. doi: 10.1016/j.neulet.2010.09.061.

Chio A., Calvo A., Dossena M., Ghiglione P., Mutani R., Mora G., (2009). "ALS in Italian professional soccer players: the risk is still present and could be soccer-specific". Amyotroph Lateral Scler 10:205-209.

Cocucci,E.,Racchetti,G.,and Mel- dolesi, J.(2009)."Shedding microvesicles: artefactsnomore". Trends Cell Biol. 19, 43–51.

Collins LM, Toulouse A, Connor TJ, Nolan YM. (2012) "Contributions of central and systemic inflammation to the pathophysiology of Parkinson's disease". Neuropharmacology., 62, 2154-68.

Colton CA, Mott RT, Sharpe H, Xu Q, Van Nostrand WE, Vitek MP. (2006) "Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD". J Neuroinflammation.;3:27.

Contestabile A., (2002). "Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro. Cerebellum". 1(1):41-55.

Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury PT Jr, (2000) "Acceleration of oligomerization, not fibrillization, is a shared property of both α-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy.", Proc Natl Acad Sci U S A.;97(2):571-6.

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, Liu K, Xu K, Strathearn KE, Liu F, Cao S, Caldwell KA, Caldwell GA, Marsischky G, Kolodner RD, Labaer J, Rochet JC, Bonini NM, Lindquist S, (2006). "A-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models.", Science.;313(5785):324-8.

Costello D.A., Lyons A., Denieffe S., Browne T.C., Cox F.F., Andlynch, M.A.(2011)."Long term potentiation is impaired in membrane glycoprotein cd200-Deficient mice: A Role for toll-Like recepto ractivation". J. Biol.Chem.286, 34722–34732.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) "Connexins regulate calcium signaling by controlling ATP release". Proc Natl Acad Sci USA 95: 15735 – 15740.

Damier P, Hirsch EC, Zhang P, Agid Y, Javoy-Agid F, (1993). "Glutathione peroxidase, glial cells and Parkinson's disease.", Neuroscience.;52(1):1-6.

De la Rosa E.J., and De Pablo F. (2000). "Cell death in early neural development: beyond the neurotrophic theory". Trends Neurosci. 23:454–458.

De Vellis J, Cole R. (2012) "Preparation of mixed glial cultures from postnatal rat brain". Methods Mol Biol., 814, 49-59.

Devi L, Raghavendran V, Prabhu BM, Avadhani NG, Anandatheerthavarada HK, (2008). "Mitochondrial import and accumulation of

α-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain.", J Biol Chem.;283(14):9089-100.

Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K, (2007). "Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model.", Nat Neurosci.;10(5):608-14.

Dissanayaka NN, Sellbach A, Matheson S, O'Sullivan JD, Silburn PA, Byrne GJ, Marsh R, Mellick GD, (2010) "Anxiety disorders in Parkinson's disease: prevalence and risk factors.", Mov Disord.;25(7):838-45.

Dubyak GR. (2012) "P2X7 receptor regulation of non-classical secretion from immune effector cells". Cell Microbiol., 14, 1697-706.

Eglitis Ma., Mezey E. (1997)" Hematopoietic Cells Differentiate Into Both Microglia And Macroglia In The Brains Of Adult Mice". Proc Natl Acad Sci US A 94:4080–4085.

Eikelenboom P, van Exel E, Veerhuis R, Rozemuller AJ, van Gool WA, Hoozemans JJ. (2012) "Innate immunity and the etiology of late-onset Alzheimer's disease". Neurodegener Dis., 10. 271-3.

El-Agnaf OM, Irvine GB, (2000), "Review: formation and properties of amyloid-like fibrils derived from α-synuclein and related proteins.", J Struct Biol.;130(2-3):300-9.

Eleuteri S, Polazzi E, Contestabile A (2008)."Neuroprotection of microglia conditioned media from apoptotic death induced by staurosporine and glutamate in cultures of rat cerebellar granule cells". Neurosci Lett; 448: 74 -78.

Eliezer D, Kutluay E, Bussell R Jr, Browne G, (2011). "Conformational properties of α-synuclein in its free and lipid-associated states.", J Mol Biol.;307(4):1061-73.

Elkabes S., Dicicco-Bloom Em., Black Ib. (1996). "Brain Microglia/ Macrophages Express Neurotrophins That Selectively Regulate Microglial Proliferation And Function". J Neurosci 16: 2508–2521.

Falchi,A.M.,Sogos,V.,Saba,F., Piras,M.,Congiu,T.,and Piludu, M. (2013). "Astrocytes shed large membrane vesicles that contain nmitochondria, lipid droplets and ATP". Histochem.CellBiol. 139, 221– 231.

Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS, Hulihan M, Maraganore D, Gwinn-Hardy K, Wszolek Z, Dickson D, Langston JW, (2004). "Comparison of kindreds with parkinsonism and α-synuclein genomic multiplications.", Ann Neurol.;55(2):174-9.

Feany MB, Bender WW, (2000) "A Drosophila model of Parkinson's disease.", Nature.;404(6776):394-8.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G (2004) "Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors". Neuron 43(5):729–743.

Ferretti MT, Bruno MA, Ducatenzeiler A, Klein WL, Cuello AC. (2012). "Intracellular Aβ-oligomers and early inflammation in a model of Alzheimer's disease". Neurobiol Aging.;3(7):1329-42.

Flierl M.A., Rittirsch D., Nadeau B. A., Chen A.J., Sarma J.V., Zetoune F.S.,Et al. (2007). "Phagocyte-Derived catecholamines enhance acute inflammatory injury". Nature 449, 721–725.

Florent ginhoux, Shawnlim, Guillaumehoeffel, Donovanlow And Tarahuber (2012). "Origin And Differentiation Of Microglia. Frontiers in cellular neuroscience". Volume 7 articolo 45.

Foltyn VN, Bendikov I, De Miranda J, Panizzutti R, Dumin E, Shleper M, Li P, Toney MD, Kartvelishvily E, Wolosker H, (2005). "Serine racemase modulates intracellular D-serine levels through an α,beta-elimination activity.", J Biol Chem.;280(3):1754-63.

Frank-Cannon T.C., Alto L.T., McAlpine F.E., Tansey M.G., (2009). "Does neuroinflammation fan the flame in neurodegenerative diseases?" Mol. Mol. Neurodegener. 4(47).

Franke H, Krügel U, Illes P. (2006) "P2 receptors and neuronal injury". Pflugers Arch., 452, 622-44.

Franke H, Verkhratsky A, Burnstock G, Illes P. (2012) "Pathophysiology of astroglial purinergic signaling". Purinergic Signal., 8, 629-57.

Frühbeis C, Fröhlich D, Kuo WP, Krämer-Albers EM. (2013) "Extracellular vesicles as mediators of neuron-glia communication". Front Cell Neurosci., 7, 182.

Fuchs J, Nilsson C, Kachergus J, Munz M, Larsson EM, Schüle B, Langston JW, Middleton FA, Ross OA, Hulihan M, Gasser T, Farrer MJ, (2007). "Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication.", Neurology.;68(12):916-22.

Gallo V., Kingsbury A., Balazs R., et al. (1987) "The role of depolarization in the survival and differentiation of cerebellar granule cells in culture". J. Neurosci., 7, 2203-2213.

Gehrmann J., Matsumoto Y., Kreutzberg G.W., (1995). "Microglia: intrinsic immuneffector cell of the brain". Brain Res. Brain Res. Rev. 20:269–287.

Geissmann F, Auffray C, Palframan R, Wirrig C, Ciocca A, Campisi L, Narni-Mancinelli E, Lauvau G, (2008). "Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses.", Immunol Cell Biol.;86(5):398-408.

Giblett, E.R., J.E. Anderson, F. Cohen, B. Pollara, and H.J. Meuwissen. (1972). "Adenosine-deaminase deficiency in twopatients with severely impaired cellular immunity". Lancet.2:1067–1069.

Go YM. and Jones DP., (2011). "Cysteine/cystine redox signaling in cardiovascular disease". Free Radic Biol Med 15;50(4):495-509.

Golgi C, "Contribuzione alla fine anatomia degli organi centrali del sistema nervoso.", 1871, Rivista Clinica di Bologna.

Gomes C, Keller S, Altevogt P, Costa J. (2007). "Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis". Neurosci Lett.;428(1):43-6.

Grada L.I., Yerburyb J.J., Turnerc B.J., Guesta W.C., Pokrishevskya E., O'Neilla M.A., Yanaia A., Silvermana J.M., Zeineddineb R., Corcoranb L., Kumitad J.R., Luheshid L.M., Yousefia M., Colemane B.M., Hille A.F., PlotkγS.S., Mackenzieg I.R. and Cashmana N.R., (2014). "Intercellular propagated misfolding of wild-type Cu/Zn superoxide dismutase occurs via exosome-dependent and -independent mechanisms". PNAS 111(9): 3620-3625.

H A Simmonds, L D Fairbanks, G S Morris, G Morgan, A R Watson, P Timms, and B Singh (1987). "Central nervous system dysfunction and

erythrocyte guanosine triphosphate depletion in purine nucleoside phosphorylase deficiency". Archives of Disease in Childhood, 62, 385-391.

Hall E.D., Oostveen J.A., Gurney M.E., (1998). "Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS". Glia 23:249–256.

Hanamsagar R, Torres V, Kielian T. (2011) "Inflammasome activation and IL-1β/IL-18 processing are influenced by distinct pathways in microglia". J Neurochem., 119, 736-48.

Hanisch UK, Kettenmann H, (2007). "Microglia: active sensor and versatile effector cells in the normal and pathologic brain.", Nat Neurosci.;10(11):1387-94.

Hanisch Uk. (2002)." Microglia As A Source And Target Of Cytokines" . Glia 40:140–155.

Hartline DK, (2011). "The evolutionary origins of glia.", Glia.;59(9):1215-36.

Haydon PG (2001) "GLIA: listening and talking to the synapse". Nat Rev 2(3):185–193.

Held H (1909) "Über die Neuroglia marginalis der menschlichen Grosshirnrinde". Psychol u Neurol 26 Rdg.-Heft: 360 – 416.

Hertz L. L. Peng, J.C. Lai. (1998) "Functional studies in cultured astrocytes". Methods, 16 , 293–310.

Hetier E, Ayala J, Denèfle P, Bousseau A, Rouget P, Mallat M, Prochiantz A, (1988). "Brain macrophages synthesize interleukin-1 and interleukin-1 mRNAs in vitro.", J Neurosci Res.;21(2-4):391-7.

Hewett JA, (2009). "Determinants of regional and local diversity within the astroglial lineage of the normal central nervous system.", J Neurochem.;110(6):1717-36.

Hibell AD, Thompson KM, Xing M, Humphrey PP, Michel AD. (2001) "Complexities of measuring antagonist potency at P2X(7) receptor orthologs". J Pharmacol Exp Ther., 296, 947-57.

Hickey Wf., Kimura H. (1988) "Perivascular Microglial Cells Of The Cns Are Bone Marrow-Derived And Present Antigen In Vivo". Science 239:290–292.

Hirsch EC, Hunot S, (2009). "Neuroinflammation in Parkinson's disease: a target for neuroprotection?", Lancet Neurol.;8(4):382-97.

Houlden H, Singleton AB, (2012). "The genetics and neuropathology of Parkinson's disease.", Acta Neuropathol. ;124(3):325-38.

Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, Chen J, (2015). "Microglial and macrophage polarization—new pro-spects for brain repair.", Nat Rev Neurol.;11(1):56-64. doi: 10.1038/nrneurol.2014.207..

Iadecola C, Nedergaard M, (2007). "Glial regulation of the cerebral microvasculature.", Nat Neurosci.;10(11):1369-76.

Ilieva H, Polymenidou M, Cleveland DW, (2009). "Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond.", J Cell Biol. ;187(6):761-72.

Imai F., Suzuki H., Oda J., Ninomiya T., Ono K., Sano H., Sawada M., (2007). "Neuroprotective effect of exogenous microglia in global brain ischemia". J. Cereb. Blood Flow Metab. 27:488–500.

Irwin DJ, Lee VM, Trojanowski JQ, (2013). "Parkinson's disease dementia: convergence of α-synuclein, tau and amyloid-β pathologies.", Nat Rev Neurosci.;14(9):626-36.

Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A, Saitoh T, (1995). "The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system.", Neuron.;14(2):467-75.

Jackson-Lewis V, Vila M, Djaldetti R, Guegan C, Liberatore G, Liu J, O'Malley KL, Burke RE, Przedborski S, (2000). "Developmental cell death in dopaminergic neurons of the *substantia nigra* of mice.", J Comp Neurol.;424(3):476-88.

Jellinger KA, (2003). "Neuropathological spectrum of synucleinopathies.", Mov Disord.;18 Suppl 6:S2-12.

Johnston J.A., Dalton M.J., Gurney M.E., Kopito R.R., (2000). "Formation of high molecular weight complexes of mutant Cu,Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis". Proc. Natl. Acad. Sci. U.S.A. 97:12571–12576.

Kahle PJ, (2008). "α-Synucleinopathy models and human neuropathology: similarities and differences.", Acta Neuropathol.;115(1):87-95.

Kamp F, Exner N, Lutz AK, Wender N, Hegermann J, Brunner B, Nuscher B, Bartels T, Giese A, Beyer K, Eimer S, Winklhofer KF, Haass C, (2010). "Inhibition of mitochondrial fusion by α-synuclein is rescued by PINK1, Parkin and DJ-1.", EMBO J.;29(20):3571-89.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A, (2011). "Physiology of microglia.", Physiol Rev. ;91(2):461-553..

Kiefer R., Streit W.J., Toyka K.V., Kreutzberg G.W., Hartung H.P., (1995). "Transforming Growth Factor-Beta 1: A Lesion-Associated Cytokine Of The Nervous System.". Int. J. Dev. Neurosci. 13:331–339.

Koenigsknecht-Talboo J, Landreth GE, (2005). "Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines.", J Neurosci.;25(36):8240-9.

Kong LY, Lai C, Wilson BC, Simpson JN, Hong JS, (1997). "Protein tyrosine kinase inhibitors decrease lipopolysaccharide-induced proinflammatory cytokine production in mixed glia, microglia-enriched or astrocyte-enriched cultures.", Neurochem Int.;30(4-5):491-7.

Lalancette-He´bert M., Gowing G., Simard A., Weng Y.C., Kriz J., (2007). "Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain". J. Neurosci. 27:2596-2605.

Lambertsen KL, Clausen BH, Babcock AA, Gregersen R, Fenger C, Nielsen HH, Haugaard LS, Wirenfeldt M, Nielsen M, Dagnaes-Hansen F, Bluethmann H, Faergeman NJ, Meldgaard M, Deierborg T, Finsen B, (2009). "Microglia protect neurons against ischemia by synthesis of tumor necrosis factor.", J Neurosci.;29(5):1319-30.

Lang PA, Merkler D, Funkner P, Shaabani N, Meryk A, Krings C, Barthuber C, Recher M, Brück W, Häussinger D, Ohashi PS, Lang KS. (2010). "Oxidized ATP inhibits T-cell-mediated autoimmunity". Eur J Immunol.;40(9):2401-8.

Lawson Lj, Perry Vh, Dri P, Gordon S. (1990). "Heterogeneity In The Distribution And Morphology Of Microglia In The Normal Adult Mouse Brain". Neuroscience.;39(1):151-70.

Lee HJ, Suk JE, Patrick C, Bae EJ, Cho JH, Rho S, Hwang D, Masliah E, Lee SJ, (2010). "Direct transfer of α-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies.", J Biol Chem.;285(12):9262-72.

Lee JK, Shin JH, Lee JE, Choi EJ. (2015). "Role of autophagy in the pathogenesis of amyotrophic lateral sclerosis". Biochim Biophys Acta.;1852(11):2517-24.

Levi G., Patrizio M., Bernardo A., et al. (1993). " Human immunodeficiency virus coat protein gp120 inhibits the beta-adrenergic regulation of astroglial and microglial functions". Proc. Natl. Acad. Sci. USA., 90, 1541-1545.

Lewis K. E., Rasmussen A. L., Bennet W., King A., West K. A., Chung R. S. and Chuah M. I., (2014). "Microglia and motor neurons during disease progression in tre SOD1 G93A mouse model of amyotrophic lateral sclerosis: changes in arginase 1 and inducible nitric oxide synthase". Journal of Neuroinflammation 11(55).

Li J., Baud O., Vartanian T., Volpe J. J. and Rosenberg P. A., (2005). "Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes". PNAS 102(28):9936-9941.

Li W, Lesuisse C, Xu Y, Troncoso JC, Price DL, Lee MK, (2004). "Stabilization of α-synuclein protein with aging and familial parkinson's disease-linked A53T mutation.", J Neurosci.;24(33):7400-9.

Li Y, Guo Y, Wang X, Yu X, Duan W, Hong K, Wang J, Han H, Li C. (2015). "Trehalose decreases mutant SOD1 expression and alleviates motor deficiency in early but not end-stage amyotrophic lateral sclerosis in a SOD1-G93A mouse model". Neuroscience.;298:12-25.

Liao B, Zhao W, Beers DR, Henkel JS, Appel SH. (2012). "Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS". Exp Neurol.;237(1):147-52.

Lieberman AP, Pitha PM, Shin HS, Shin ML, (1998). "Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus.", Proc Natl Acad Sci U S A.;86(16):6348-52.

Lisi L, Laudati E, Navarra P, Dello Russo C. (2014). "The mTOR kinase inhibitors polarize glioma-activated microglia to express a M1 phenotype". J Neuroinflammation.;11:125.

Liu G, Zhang C, Yin J, Li X, Cheng F, Li Y, Yang H, Uéda K, Chan P, Yu S, (2009). "α-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity.", Neurosci Lett.;454(3):187-92.

Loeb V, Yakunin E, Saada A, Sharon R, (2010). "The transgenic overexpression of α-synuclein and not its related pathology associates with complex I inhibition.", J Biol Chem.;285(10):7334-43.

Lue LF, Schmitz C, Walker DG. (2015). "What happens to microglial TREM2 in Alzheimer's disease: Immunoregulatory turned into immunopathogenic?". Neuroscience.;302:138-50.

Madinier A, Bertrand N, Mossiat C, Prigent-Tessier A, Beley A, Marie C, Garnier P, (2009). "Microglial involvement in neuroplastic changes following focal brain ischemia in rats.", PLoS One.;4(12):e8101.

Mansouri A, Min W, Cole CJ, Josselyn SA, Henderson JT, van Eede M, Henkelman RM, Ackerley C, Grunebaum E, Roifman CM. (2012) "Cerebellar abnormalities in purine nucleoside phosphorylase deficient mice". Neurobiol Dis., 47, 201-9.

Maragakis NJ, Rothstein JD, (2006). "Mechanisms of Disease: astrocytes in neurodegenerative disease.", Nat Clin Pract Neurol.;2(12):679-89.

Markert ML. (1991). "Purine nucleoside phosphorylase deficiency". Immunodefic Rev., 3, 45-81.

Martin LJ, Pan Y, Price AC, Sterling W, Copeland NG, Jenkins NA, Price DL, Lee MK, (2006). "Parkinson's disease α-synuclein transgenic mice develop neuronal mitochondrial degeneration and cell death.", J Neurosci.;26(1):41-50.

Martineau M, Shi T, Puyal J, Knolhoff AM, Dulong J, Gasnier B, Klingauf J, Sweedler JV, Jahn R, Mothet JP (2013) "Storage and uptake of D-serine into astrocytic synaptic-like vesicles specify gliotransmission". J Neurosci 33: 3413 – 3423.

Martinez-Martin P, Rodriguez-Blazquez C, Kurtis MM, Chaudhuri KR; NMSS Validation Group, (2011). "The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease.", Mov Disord.;26(3):399-406.

Miao L., and St Clair DK., (2009). "Regulation of superoxide dismutase genes: implications in disease". Free Radic Biol Med 47: 344–356.

Michel AD, Kaur R, Chessell IP, Humphrey PP. (2000) "Antagonist effects on human P2X(7) receptor-mediated cellular accumulation of YO-PRO1". Br J Pharmacol., 130, 513-20.

Milanese M, Bonifacino T, Zappettini S, Usai C, Tacchetti C, Nobile M, Bonanno G. (2009). "Glutamate release from astrocytic gliosomes under physiological and pathological conditions". Int Rev Neurobiol.;85:295-318.

Minghetti L., Levi G. (1998). "Microglia As Effector Cells In Brain Damage And Repair: Focus On Prostanoids And Nitric Oxide". Prog Neurobiol 54:99–125.

Molofsky AV, Krencik R, Ullian EM, Tsai HH, Deneen B, Richardson WD, Barres BA, Rowitch DH, (2012). "Astrocytes and disease: a neurodevelopmental perspective.", Genes Dev.;26(9):891-907.

Montana V, Liu W, Mohideen U, Parpura V (2009). "Single molecule measurements of mechanical interactions within ternary SNARE complexes and dynamics of their disassembly: SNAP25 vs. SNAP23". J Physiol 587: 1943 – 1960.

Mosharov EV, Staal RG, Bové J, Prou D, Hananiya A, Markov D, Poulsen N, Larsen KE, Moore CM, Troyer MD, Edwards RH, Przedborski S, Sulzer D, (2006). "A-synuclein overexpression increases cytosolic catecholamine concentration.", J Neurosci.;26(36):9304-11.

Mothet JP, Pollegioni L, Ouanounou G, Martineau M, Fossier P, Baux G (2005). "Glutamate receptor activation triggers a calciumdependent and

SNARE protein-dependent release of the gliotransmitter D-serine". Proc Nat Acad Sci USA 102(15) 5606–5611.

Mustafa AK, Ahmad AS, Zeynalov E, Gazi SK, Sikka G, Ehmsen JT, Barrow RK, Coyle JT, Snyder SH, Doré S, (2010). "Serine racemase deletion protects against cerebral ischemia and excitotoxicity.", J Neurosci.;30(4):1413-6.

Nagai M, Re DB, Nagata T, Chalazonitis A, Jessell TM, Wichterle H, Przedborski S, (2007)."Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons., Nat Neurosci.;10(5):615-22.

Nageotte J (1910). "Phenomenes de secretion dans le protoplasma des cellules nevrogliques de la substance grise". C R Soc Biol (Paris) 68: 1068 – 1069.

Nakamura K, Nemani VM, Azarbal F, Skibinski G, Levy JM, Egami K, Munishkina L, Zhang J, Gardner B, Wakabayashi J, Sesaki H, Cheng Y, Finkbeiner S, Nussbaum RL, Masliah E, Edwards RH, (2011)."Direct membrane association drives mitochondrial fission by the Parkinson disease-associated protein α-synuclein.", J Biol Chem.;286(23):20710-26.

Napoli I., Neumann H. (2009). "Microglial Clearance Function In Health And Disease". Neuroscience 158:1030–1038.

Nathan C, Calingasan N, Nezezon J, Ding A, Lucia MS, La Perle K, Fuortes M, Lin M, Ehrt S, Kwon NS, Chen J, Vodovotz Y, Kipiani K, Beal MF, (2005). "Protection from Alzheimer's-like disease in the mouse by genetic ablation of inducible nitric oxide synthase.", J Exp Med.;202(9):1163-9.

Neumann J., Gunzer M., Gutzeit H.O., Ullrich O., Reymann K.G., Dinkel K., (2006). Microglia provide neuroprotection after ischemia. FASEB J. 20:714-716.

Nishiya T, Uehara T, Nomura Y, (1995). "Herbimycin A suppresses NF-kappa B activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in C6 glioma cells.", FEBS Lett.;371(3):333-6.

Norris EH, Giasson BI, Lee VM, (2004). "A-synuclein: normal function and role in neurodegenerative diseases.", Curr Top Dev Biol.;60:17-54.

O'Sullivan SS, Williams DR, Gallagher DA, Massey LA, Silveira-Moriyama L, Lees AJ, (2008)."Nonmotor symptoms as presenting complaints in Parkinson's disease: a clinicopathological study.", Mov Disord.;23(1):101-6.

Pahan K, Sheikh FG, Namboodiri AM, Singh I, (1997). "Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages.", J Clin Invest.;100(11):2671-9.

Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH, (2006). "Glia-derived D-serine con-trols NMDA receptor activity and synaptic memory.", Cell.;125(4):775-84.

Pankratz N, Wilk JB, Latourelle JC, DeStefano AL, Halter C, Pugh EW, Doheny KF, Gusella JF, Nichols WC, Foroud T, Myers RH; (2009). "Genomewide association study for susceptibility genes contributing to familial Parkinson disease.", Hum Genet.;124(6):593-605.

Paolicelli R.C.,Bolasco G.,Pagani F.,Maggi L.,Scianni M.,Panzanelli P.,Et al.(2011).Synaptic pruning by microglia is necessary for normal brain development. Science 333, 1456–1458.

Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P, (2009)."A-synuclein overexpression and aggregation exacerbates impairment of mitochondrial functions by augmenting oxidative stress in human neuroblastoma cells.", Int J Biochem Cell Biol.;41(10):2015-24.

Parpura V, Haydon PG,(2009). "Astrocytes in (patho) physiology of the nervous system.", Springer, New York.

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF Jr, Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R, Verkhratsky A, (2012)."Glial cells in (patho)physiology.", J Neurochem.;121(1):4-27.

Pascual O, Ben Achour S, Rostaing P, Triller A, Bessis A. (2012). "Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission". Proc Natl Acad Sci U S A.;109(4):E197-205.

Pasinelli P. and Brown R.H., (2006). "Molecular biology of amyotrophic lateral sclerosis: insights from genetics". Nature Publishing Group 7:710-723.

Paul P, de Belleroche J.(2012). "The role of D-amino acids in amyotrophic lateral sclerosis pathogenesis: a review". Amino Acids.;43(5):1823-31.

Paul P, de Belleroche J. (2014). "The role of D-serine and glycine as co-agonists of NMDA receptors in motor neuron degeneration and amyotrophic lateral sclerosis (ALS)". Front Synaptic Neurosci. 2014 Apr 16;6:10.

Pellerin L, Bouzier-Sore AK, Aubert A, Serres S, Merle M, Costalat R, Magistretti PJ,(2007). "Activity-dependent regulation of energy metabolism by astrocytes: an update.", Glia.;55(12):1251-62.

Petrova TV, Akama KT, Van Eldik LJ, (1999). "Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy-Delta12,14-prostaglandin J2.", Proc Natl Acad Sci U S A.;96(8):4668-73.

Philips T, Robberecht W. (2011). "Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease". Lancet Neurol.;10(3):253-63.

Polazzi E, Monti B, (2010). "Microglia and neuroprotection: from in vitro studies to therapeutic applications.", Prog Neurobiol.;92(3):293-315.

Polazzi E., Gianni T. and Contestabile A., (2001). "Microglial Cells Protect Cerebellar Granule Neurons from Apoptosis: Evidence for Reciprocal Signaling". GLIA 36:271-280.

Polazzi E., Mengoni I., Caprini M., Peña-Altamira L.E., Kurtys E., Monti B., (2013)." Copper-Zinc Superoxide Dismutase (SOD1) Is Released by Microglial Cells and Confers Neuroprotection against 6-OHDA Neurotoxicity". Neurosignals 21: 112-128.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL, (1997). "Mutation in the α-synuclein gene identified in families with Parkinson's disease.", Science.;276(5321):2045-7.

Potolicchio I, Carven GJ, Xu X, Stipp C, Riese RJ, Stern LJ, Santambrogio L. "2005". Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. J Immunol;175:2237–2243.

Puentes F, Malaspina A, van Noort JM, Amor S. (2016). "Non-neuronal cells in ALS: Role of glial, immune cells and blood-CNS barriers". Brain Pathol.

Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT, (2007). "Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration.", Glia.;55(5):453-62.

Ransohoff R. M., Perry V. H. (2009). "Microglial physiology: unique stimuli, specialized responses". Annu. Rev. Immunol. 27 119–145.

Ransohoff RM, Brown MA. (2012) "Innate immunity in the central nervous system". J Clin Invest., 122, 1164-71.

Rappold PM, Tieu K, (2010). "Astrocytes and therapeutics for Parkinson's disease.", Neurotherapeutics.;7(4):413-23.

Redmanna Matthew, Willayat Y. Wania, Laura Volpicelli-Daleyc, Victor Darley-Usmara, Jianhua Zhanga (2017) "Trehalose does not improve neuronal survival on exposure to alphasynuclein pre-formed fibrils". Redox Biology 11, 429–437.

Rezaie P, Male D, (2002). "Mesoglia & microglia--a historical review of the concept of mononuclear phagocytes within the central nervous system.", J Hist Neurosci.;11(4):325-74.

Rezaie, P., Dean, A., Male, D., Ulfig, N., (2005b). "Microglia In The Cerebral Wall Of The Human Telencephalon At Second Trimester". Cereb. Cortex 15, 938–949.

Richards AB, Krakowka S, Dexter LB, Schmid H, Wolterbeek AP, Waalkens-Berendsen DH, Shigoyuki A, Kurimoto M (2002) "Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies". Food Chem Toxicol 40:871–898.

Robak T, Lech-Maranda E, Korycka A, Robak E. (2006). "Purine nucleoside analogs as immunosuppressive and antineoplastic agents: mechanism of action and clinical activity". Curr Med Chem., 13, 3165-89.

Rodriguez-Navarro JA, Rodriguez L, Casarejos MJ, Solano RM, Gomez A, Perucho J, Cuervo AM, Garcia de Yebenes J, Mena MA (2010). "Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation". Neurobiol Dis 39:423–438.

Rojanathammanee L, Murphy EJ, Combs CK, (2011). "Expression of mutant α-synuclein modulates microglial phenotype *in vitro*.", J Neuroinflammation.;8:44. doi: 10.1186/1742-2094-8-44.

Rosen D.R., Siddique T., Patterson D., Figlewicz D.A., Sapp P., Hentati A., Donaldson D., Goto J., O'Regan P., Deng H.X., Rhamani Z., Krizus A., McKenna-Yasek D., Cayabyab A., Gaston S.M., Berger R., Tanzi R.E., Halperin J.J., Herzfeldt B., Van den Bergh R., Hung W.Y., Uccello T., Deng G., Mulder D.W., Smyth C., Laing N.G., Soriano E., Pericak-Vance M.A., Haines J., Rouleau G.A., Gusella J.S., Horvitz H.R., Brown R.H. Jr., (1993). "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis". Nature 362:59-62.

Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC, (2007). "Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein". J Biol Chem.;282(8):5641-52.

Sau D, De Biasi S, Vitellaro-Zuccarello L, Riso P, Guarnieri S, Porrini M, Simeoni S, Crippa V, Onesto E, Palazzolo I, Rusmini P, Bolzoni E, Bendotti C, Poletti A., (2007). "Mutation of SOD1 in ALS: a gain of a loss of function". Hum Mol Genet. 16(13):1604-1618.

Schell MJ, Molliver ME, Snyder SH, (1995). "D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release.", Proc Natl Acad Sci U S A.;92(9):3948-52.

Schrag A, Quinn N,(2000). "Dyskinesias and motor fluctuations in Parkinson's disease. A community-based study.", Brain.;123 (Pt 11):2297-305.

Schroder Kate, Hertzog Paul J, Ravasi Timothy, Hume David A. (2017). "Interferon-: an overview of signals, mechanisms and functions". Journal of Leukocyte Biology Vol.75, No.2 , pp:163-189.

Serpell LC, Berriman J, Jakes R, Goedert M, Crowther RA, (2002)."Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-beta conformation.", Proc Natl Acad Sci U S A.;97(9):4897-902.

Shie FS, Chen YH, Chen CH, Ho IK. (2011)." Neuroimmune pharmacology of neurodegenerative and mental diseases". J Neuroimmune Pharmacol., 6, 28-40.

Shigetomi E, Bowser DN, Sofroniew MV, Khakh BS (2008). "Two forms of astrocyte calcium excitability have distinct effects on NMDA receptor-mediated slow inward currents in pyramidal neurons". J Neurosci 28(26):6659–6663.

Shimizu H, Watanabe E, Hiyama TY, Nagakura A, Fujikawa A, Okado H, Yanagawa Y, Obata K, Noda M, (2007). "Glial Nax channels control lactate signaling to neurons for brain [Na+] sensing.",;54(1):59-72.

Shleper M, Kartvelishvily E, Wolosker H, (2005). "D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices.", J Neurosci.;25(41):9413-7.

Silva RG, Santos DS, Basso LA, Oses JP, Wofchuk S, Portela LV, Souza DO (2004). "Purine nucleoside phosphorylase activity in rat cerebrospinal fluid". Neurochem Res., 29, 1831-5.

Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M, (2003). "Signaling at the gliovascular interface.", J Neurosci.;23(27):9254-62.

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K,(2003). "α-Synuclein *locus* triplication causes Parkinson's disease.", Science.;302(5646):841.

Singleton AB, Farrer MJ, Bonifati V, (2013)."The genetics of Parkinson's disease: progress and therapeutic implications.", Mov Disord.;28(1):14-23.

Skaper SD, Debetto P, Giusti P. (2010). The P2X7 purinergic receptor: from physiology to neurological disorders. FASEB J., 24. 337-45.

Skaper SD. (2011). Ion channels on microglia: therapeutic targets for neuroprotection. CNS Neurol Disord Drug Targets., 10, 44-56.

Snyder, F.F., J.P. Jenuth, E.R. Mably, and R.K. Mangat. (1997). Point mutations at the purine nucleoside phosphorylase locus impair thymocyte differentiation in the mouse. Proc. Natl. Acad. Sci. USA. 94:2522–2527.

Stefanova N, Klimaschewski L, Poewe W, Wenning GK, Reindl M, (2011). "Glial cell death induced by overexpression of α-synuclein.", J Neurosci Res.;65(5):432-8.

Steiner JA, Angot E, Brundin P (2011). "A deadly spread: cellular mechanisms of α-synuclein transfer.", Cell Death Differ.;18(9):1425-33.

Stence N., Waite M., Dailey Me. (2001). Dynamics Of Microglial Activation: A Confocal Time-Lapse Analysis In Hippocampal Slices. Glia 33: 256–266.

Stichel CC, Zhu XR, Bader V, Linnartz B, Schmidt S, Lübbert H, (2007). "Mono- and double-mutant mouse models of Parkinson's disease display severe mitochondrial damage.", Hum Mol Genet.;16(20):2377-93.

Streit WJ, (2004). "Microglia and Alzheimer's disease pathogenesis.", J Neurosci Res.;77(1):1-8.

Streit Wj, Walter Sa, Pennell Na. (1999). Reactive Microgliosis. Prog Neurobiol 57:563–581.

Streit WJ, Xue QS, (2009). " Life and death of microglia.", J Neuroimmune Pharmacol.;4(4):371-9.

Suadicani SO, Brosnan CF, Scemes E (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J Neurosci 26: 1378 – 1385.

Szabo M., and Gulya K., (2013). Development Of The Microglial Phenotype In Culture Neuroscience 241 280–295.

Takahashi, K., (2001). Development And Differentiation Of Macrophages And Related Cells: Historical Review And Current Concepts. J. Clin. Exp. Hemat. 41, 1–33.

Takato Takenouchi, Masaaki Nakai, Yoshifumi Iwamaru, Shuei Sugama, Mitsutoshi Tsukimoto, Masayo Fujita, Jianshe Wei, Akio Sekigawa, Mitsuru Sato, Shuji Kojima, Hiroshi Kitani and ;akoto Hashimoto (2009). The Activation of P2X7 Receptor Impairs Lysosomal Functions and Stimulates the Release of Autophagolysosomes in Microglial Cells. The Journal of Immunology, 182:2051-2062.

Takeda K., Kaisho T., Akira S. (2003). Toll-Like Receptors. Annu Rev Immunol 21:335–376.

Takenouchi T, Fujita M, Sugama S, Kitani H, Hashimoto M. (2009). The role of the P2X7 receptor signaling pathway for the release of autolysosomes in microglial cells. Autophagy., 5, 723-4.

Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, Kurosawa M, Nekooki M, Nukina N (2004).Trehalose alleviates polyglutaminemediated pathology in a mouse model of Huntington disease. Nat Med 10:148–154.

Tanida I, Ueno T, Kominami E. (2004). LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol.;36(12):2503-18.

Thery,C.(2011).Exosomes: secreted vesicles and intercellular communications. F1000 Biol.Rep. 3, 15.

Thored P., Heldmann U., Gomes-Leal W., Gisler R., Darsalia V., Taneera J.,Nygren Town T., Nikolic V., Tan J. (2005). The Microglial ''Activation'' Continuum: From Innate To Adaptive Responses. J Neuroinflammation 2:24.

Thored, P., Heldmann, U., Gomes-Leal, W., Gisler, R., Darsalia, V., Taneera, J., Nygren, J.M., Jacobsen S.E., Ekdahl C.T., Kokaia Z., Lindvall O. (2009). Long-Term Accumulation Of Microglia With Proneurogenic Phenotype Concomitant With Persistent Neurogenesis In Adult Subventricular Zone After Stroke. Glia 57, 835–849.

Toro A, Grunebaum E. (2006). TAT-mediated intracellular delivery of purine nucleoside phosphorylase corrects its deficiency in mice. J Clin Invest., 116, 2717-26.

Tremblay M.-È.,Stevens B.,Sierra A., Wake H.,Bessis A.,and Nimmerjahn A.(2011).The role of microglia in the healthy brain. J. Neurosci. 31, 16064–16069.

Ullian EM, Harris BT, Wu A, Chan JR, Barres BA, (2004). "Schwann cells and astrocytes induce synapse formation by spinal motor neurons in culture.", Mol Cell Neurosci.;25(2):241-51.

Unichenko P, Dvorzhak A, Kirischuk S (2013) Transporter-mediated replacement of extracellular glutamate for GABA in the developing murine neocortex. Eur J Neurosci 38: 3580 – 3588.

Ushio-Fukai M., (2009). Compartmentalization of redox signaling through NADPH oxidase-derived ROS. Antioxid Redox Signal 11: 1289–1299.

Uversky VN, Fink AL, (2002). "Amino acid determinants of α-synuclein aggregation: putting together pieces of the puzzle.", FEBS Lett.;522(1-3):9-13.

Van Damme P, Dewil M, Robberecht W, Van Den Bosch L, (2005)."Excitotoxicity and amyotrophic lateral sclerosis.", Neurodegener Dis.;2(3-4):147-59.

Vekrellis K, Rideout HJ, Stefanis L, (2004). "Neurobiology of α-synuclein.", Mol Neurobiol.;30(1):1-21.

Venda LL, Cragg SJ, Buchman VL, Wade-Martins R, (2010). "α-Synuclein and dopamine at the crossroads of Parkinson's disease.", Trends Neurosci.;33(12):559-68.

Verderio C, Cagnoli C, Bergami M, Francolini M, Schenk U, Colombo A, Riganti L, Frassoni C, Zuccaro E, Danglot L, Wilhelm C, Galli T, Canossa M, Matteoli M. (2012) TI-VAMP/VAMP7 is the SNARE of secretory lysosomes contributing to ATP secretion from astrocytes. Biol Cell., 104, 213-28.

Verderio,C.,Muzio,L.,Turola,E., Bergami, A.,Novellino,L.,Ruffini,F., et al.(2012).Myeloidmicrovesicles areamarkerandtherapeutictarget for neuroinflammation. Ann.Neurol. 72, 610–624.

Verderio,C.,Muzio,L.,Turola,E., Bergami, A.,Novellino,L.,Ruffini,F., et al.(2012). Myeloid microvesicles are a marker and therapeutic target for neuroinflammation. Ann.Neurol. 72, 610–624.

Verkhratsky A, Olabarria M, Noristani HN, Yeh CY, Rodriguez JJ, (2010)."Astrocytes in Alzheimer's disease.", Neurotherapeutics.;7(4):399-412.

Verkhratsky A. Patching (2006). The Glia Reveals The Functional Organisation Of The Brain. Pflügers Arch 453: 411–420.

Volterra A, Meldolesi J (2005). Astrocytes, from brain glue to 9communication elements: the revolution continues. Nat Rev 6(8):626–640.

Volterra A., Magistretti P. J., and Haydon P. G., "Tripartite Synapse: Glia in Synaptic Transmission", Eds., vol. 1, pp. 3–23, Oxford University Press, Oxford, UK.

Wake H., Moorhouse A.J., Miyamoto A. and Nabekura J., (2013). Microglia: actively surveying and shaping neuronal circuit structure and function. Trends in Neurosciences 36(4).

Walton, N.M., Sutter, B.M., Laywell, E.D., Levkoff, L.H., Kearns, S.M., Marshall Ii, G.P., Scheffler, B., Steindler, D.A., (2006). Microglia Instruct Subventricular Zone Neurogenesis. Glia 54, 815–825.

Wang J., Xu G. and Borchelt D. R., (2002). High Molecular Weight Complexes of Mutant Superoxide Dismutase 1: Age-Dependent and Tissue-Specific Accumulation. Neurobiology of Disease 9:139-148.

Wang L, Sharma K, Grisotti G, Roos RP, (2009). "The effect of mutant SOD1 dismutase activity on non-cell autonomous degeneration in familial amyotrophic lateral sclerosis.", Neurobiol Dis.,35(2):234-40.

Wang,G.,Dinkins,M.,He,Q.,Zhu,G., Poirier,C.,Campbell,A.,etal.(2012). Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR- 4): potential mechanism of apoptosis induction in Alzheimer disease(AD). J. Biol.Chem. 287, 21384–21395.

Weintraub D, Koester J, Potenza MN, Siderowf AD, Stacy M, Voon V, Whetteckey J, Wunderlich GR, Lang AE, (2010)."Impulse control disorders in Parkinson disease: a cross-sectional study of 3090 patients.", Arch Neurol.;67(5):589-95.

Weisman GA, Camden JM, Peterson TS, Ajit D, Woods LT, Erb L. (2012). P2 receptors for extracellular nucleotides in the central nervous system: role of P2X7 and P2Y$_2$ receptor interactions in neuroinflammation. Mol Neurobiol.,46, 96-113.

Wills AM, Cronin S, Slowik A, Kasperaviciute D, Van Es MA, Morahan JM, Valdmanis PN, Meininger V, Melki J, Shaw CE, Rouleau GA,

Fisher EM, Shaw PJ, Morrison KE, Pamphlett R, Van den Berg LH, Figlewicz DA, Andersen PM, Al-Chalabi A, Hardiman O, Purcell S, Landers JE, Wolfgang J Streit (2002). Microglia As Neuroprotective, Immunocompetent Cells Of The Cns. Glia. 40(2):133-9.

Wilms, H., Rosenstiel, P., Sievers, J., Deuschl, G., Lucius, R., (2001). Cerebrospinal fluid from patients with neurodegenerative and neuroinflammatory diseases: no evidence for rat glial activation in vitro. Neurosci. Lett. 314:107–110.

Wood-Kaczmar A, Gandhi S, Wood NW, (2006). "Wood-Kaczmar A, Gandhi S, Wood NW, "Understanding the molecular causes of Parkinson's disease.", Trends Mol Med.;12(11):521-8.

Wu S, Barger SW, (2004). "Induction of serine racemase by inflammatory stimuli is dependent on AP-1.", Ann N Y Acad Sci.; 1035:133-46.

Yamakura F and Kawasaki H, (2010). Post-translational modifications of superoxide dismutase. Biochim Biophys Acta 1804: 318–325.

Yamanaka K, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, Misawa H, Cleveland DW, (2008). "Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis.", Nat Neurosci.;11(3):251-3.

Yanagisawa D., Kitamura Y., Takata K., Hide I., Nakata Y., Taniguchi T., (2008). Possible involvement of P2X7 receptor activation in microglial neuroprotection against focal cerebral ischemia in rats. Biol. Pharm. Bull. 31:1121–1130.

Young MT, Pelegrin P, Surprenant A. (2007) Amino acid residues in the P2X7 receptor that mediate differential sensitivity to ATP and BzATP. Mol Pharmacol., 71, 92-100.

Young, P., J.M. Leeds, M.B. Slabaugh, and C.K. Mathews. (1994). Ribonucleotide reductase: evidence for specific association with HeLa cell mitochondria. Biochem. Biophys. Res. Commun. 203:46–52

Yuste JE, Tarragon E, Campuzano CM, Ros-Bernal F. (2015). Implications of glial nitric oxide in neurodegenerative diseases. Front Cell Neurosci.;9:322.

Zarranz JJ, Alegre J, Gómez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atarés B, Llorens V, Gomez Tortosa E, del Ser T, Muñoz DG, de Yebenes JG, (2004). "The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia.", Ann Neurol.;55(2):164-73.

Zelko IN, Mariani TJ, and Folz RJ, (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33: 337–349.

Zhang J, Ney PA, (2008). "NIX induces mitochondrial autophagy in reticulocytes.", Autophagy;4(3):354-6.

Zhao W, Beers DR, Appel SH. (2013). Immune-mediated mechanisms in the pathoprogression of amyotrophic lateral sclerosis. J Neuroimmune Pharmacol.;8(4):888-99.

Zielasek J, Hartung HP. Molecular mechanisms of microglial activation. (1996) Adv Neuroimmunol., 6, 191-22.

Zimprich A, Benet-Pagès A, Struhal W, Graf E, Eck SH, Offman MN, Haubenberger D, Spielberger S, Schulte EC, Lichtner P, Rossle SC, Klopp N, Wolf E, Seppi K, Pirker W, Presslauer S, Mollenhauer B, Katzenschlager R, Foki T, Hotzy C, Reinthaler E, Harutyunyan A, Kralovics R, Peters A, Zimprich F, Brücke T, Poewe W, Auff E, Trenkwalder C, Rost B, Ransmayr G, Winkelmann J, Meitinger T, Strom TM, (2011). "A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease.", Am J Hum Genet.;89(1):168-75.

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., Cohen, H., Kipnis, J.,Schwartz, M., (2006). Immune Cells Contribute To The Maintenance Of Neurogenesis And Spatial Learning Abilities In Adulthood. Nat. Neurosci. 9, 268–275.