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Microglial involvement in brain physiopathology: *in vitro* studies using rat primary cultures

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TABLE OF CONTENTS

Page

ABSTRACT

Microglial involvement in neurological disorders is well-established, being microglial activation not only associated with neurotoxic consequences, but also with neuroprotective effects.

The studies presented here, based on microglia rat primary cell cultures and mainly on microglial conditioned medium (MCM), show insights into the mechanism of Superoxide dismutase 1 (SOD1) and Apolipoprotein E (ApoE) secretion by microglia as well as their neuroprotective effect towards primary cerebellar granule neurons (CGNs) exposed to the dopaminergic toxin 6-hydroxydopamine (6-OHDA).

SOD1 and ApoE are released respectively through non-classical lysosomal or the classical ER/Golgi-mediated secretion pathway. Microglial conditioned medium, in which SOD1 and ApoE accumulated, protected CGNs from degeneration and these effects were replicated when exogenous SOD1 or ApoE was added to a non-conditioned medium. SOD1 neuroprotective action was mediated by increased cell calcium from an external source. ApoE release is negatively affected by microglia activation, both with lipopolysaccharide (LPS) and Benzoylbenzoyl-ATP (Bz-ATP) but is stimulated by neuronal-conditioned medium as well as in microglia-neurons co-culture conditions. This neuronal-stimulated microglial ApoE release is differently regulated by activation states (i.e. LPS vs ATP) and by 6-hydroxydopamine-induced neurodegeneration. In co-culture conditions, microglial ApoE release is essential for neuroprotection, since microglial ApoE silencing through siRNA abrogated protection of cerebellar granule neurons against 6-OHDA toxicity. Therefore, these molecules could represent a target for manipulation aimed at promoting neuroprotection in brain diseases.

Considering a pathological context, and the microglial ability to adopt a neuroprotective or neurotoxic profile, we characterize the microglial M1/M2 phenotype in transgenic rats (McGill-R-Thy1-APP) which reproduce extensively the Alzheimer's-like amyloid pathology. Here, for the first time, cortical, hippocampal and cerebellar microglia of wild type and transgenic adult rats were compared, at both early and advanced stages of the pathology. In view of possible therapeutic translations, these findings are relevant to test microglial neuroprotection, in animal models of neurodegenerative diseases.

CHAPTER 1:

INTRODUCTION

1.1 MICROGLIAL CELLS: FROM FIRST CHARACTERIZATION TO LATEST DISCOVERIES

The "history of physiology" is rich of examples of cell types whose function has been rapidly discovered after their first isolation or characterization. This is not the case of microglial cells, or at least, not completely. First discovered in the early 1900, after several decades of studies, there is still wide space for new insights concerning the role of this heterogeneous cell population, especially in pathological situations.

Actually, following the early description of neuroglia by Virchow in the mid nineteenth century, other contemporary pathologists and psychiatrists, including Nissl and Alzheimer, are noteworthy for their remarkable insights on the nature of microglia. In particular they commented on the possibility that during its development the CNS was populated by cells of non-neuroectodermal origin (Kettenmann and Ransom, 2012).

Speculation abounded as to the source of these invading cells, but particular attention was focused with increased consistency on the possibility that mesodermally derived cells were the invaders.

This led to the formulation by Cajal of *el tercer elemento*, the third element of the CNS, referring to a group of cells that was morphologically distinct from both first and second elements (neurons and astrocytic neuroglia). However it was the Spanish neuroanatomist del Rio-Hortega who, in 1932, earned the title "father of microglia biology" for his further distinction of Cajal's third element into oligodendrocytes and microglia and for his first systematic investigation on microglial cells, which remains quite relevant even today (Rock et al., 2004; Kettenmann and Ransom, 2012).

However, following this era of intense scientific investigations, the field of research on microglia experienced an eclipse that lasted half a century (Rock et al., 2004). In recent history, the debate over microglia ontogeny has been followed by another fervent discussion, this one focusing on the functional significance of activated microglial cells, that is, whether activation of microglial cells is a beneficial or detrimental process (Kettenmann and Ransom, 2012). More precisely in the past 15-20 years a surprising awakening of interest is witnessed by thousands of published articles containing the term "microglia" in their title. This rebirth of interest is due to the well-established evidence of microglial involvement in neurodegenerative diseases (Rock et al., 2004), being microglial activation and neuroinflammation common features of these neuropathologies (Polazzi and Monti, 2010).

1.1.1 Current consensus on microglial ontogeny

Two main issues characterized a longstanding debate concerning the origin of microglia: the mesodermal versus neuro-ectodermal derivation and when and how do microglia populate the Central Nervous System.

Nowadays it seems that both issues have been largely resolved as it is widely accepted that (i) unlike astrocytes, neurons and oligodendrocytes, which are derived from neuroectoderm, both perivascular and parenchymal microglia are from a "myeloid-monocytic" lineage and therefore derived from hemangioblastic mesoderm and (ii) microglia become part of the CNS parenchyma early during embryonic development at about the time neurulation has been completed (Kettenmann and Ransom, 2012).

Microglial precursor cells are an important component of the CNS during both embryonic and postnatal development. Cells described as "fetal macrophages" (Takahashi et al., 1989) populate the developing neuroectoderm as early as embryonic day 8 in rodents and during the first trimester in humans (Alliot et al., 1999; Monier et al., 2007; Ginhoux et al., 2010) and are considered the earliest detectable microglial precursor cells because they can be visualized using lectin histochemical markers that specifically label microglia (Sorokin et al., 1992). Interestingly, this fetal macrophages can be found in the primitive neuroectoderm before it becomes vascularized (Chan et al., 2007; Monier et al., 2007) which excludes the possibility that blood-borne monocytes are direct microglial precursors. When the embryonic CNS develops toward the perinatal stage, and various neural cell types mature and differentiate, fetal macrophages, characterized by a rounded morphology, also turn into a more differentiated embryonic microglia with short processes (Kettenmann and Ransom, 2012).

In rodents, during perinatal stages (at about embryonic day 20), islands of so called ameboid microglial cells appear (Ling and Wong, 1993) as aggregated clusters of rounded cells in specific anatomical locations, particularly in the supraventricular corpus callosum (Ling and Wong, 1993; Hurley et al., 1999). After birth, ameboid microglia within these clusters undergo mitosis, and these supraventricular clusters of proliferating cells were recognized and termed them *fountains of microglia* by early microglial researchers (Kettenmann et al., 2011)

Contemporary neurobiologists might prefer the term microglial progenitors cells instead of ameboid microglia in order to emphasize their status of immature precursor cells.

During the first two postnatal weeks, microglial progenitor cells migrate from the corpus callosum into the overlying cerebral cortex, differentiating into fully ramified microglia.

This perinatal microgliogenesis facilitates microglial colonization of the forebrain, which presents its most expansive growth during the postnatal period (Kettenmann and Ransom, 2012).

In adult physiological conditions there is a minimal replacement of microglia from exogenous sources, such as the bone marrow (Ajami et al., 2011). Furthermore microglia have the greatest mitotic potential among all parenchymal cells in the CNS and are therefore capable of self-renewal. However, microglial proliferation in the normal CNS occurs at a very low rate, indicating low turnover as well as a long cells lifespan (Lawson et al., 1990). Nonetheless, a small fraction of microglial cells may undergo replacement by bone marrow-derived precursors via perivascular cells, the mononuclear phagocytes that reside in the Virchow-Robin (perivascular) spaces surrounding medium and small-sized cerebral vessels (Kettenmann and Ransom, 2012).

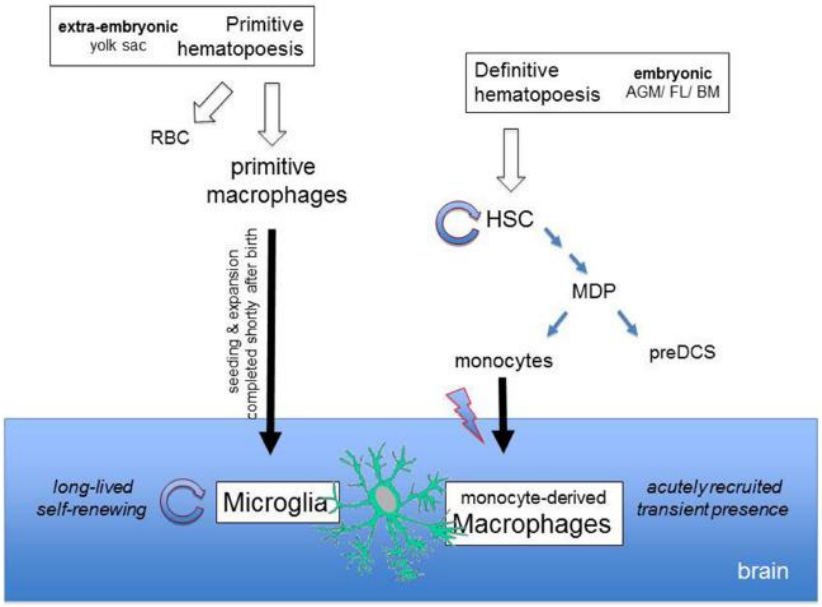

Fig.1. Differential origins of microglia and monocyte-derived brain macrophages (Jung and Schwartz, 2012).

While the concept of physiologic infiltration of bone marrow-derived progenitors in the intact CNS is somehow disputed, there is no doubt that these precursors populate the brain during injuries and diseases (Eglitis and Mezey, 1997; Flugel et al., 2001; Beck et al., 2003; Ladeby et al., 2005). On occasion, perivascular cells may penetrate the perivascular basement membrane, enter the parenchyma and differentiate into process-bearing microglia, an hypothesis supported by studies using bone marrow chimeras and localization of major histocompatibility antigens (Hickey and Kimura, 1988; Streit et al., 1989).

As concluding remark, the identification of the origins (tissue and lineage) of resident parenchymal microglia have an extreme importance for therapeutic approaches

that may use these cells as a vehicle for delivering key molecules capable of improving the nervous system in case of injury and disease (Polazzi and Monti, 2010).

1.2 WHAT IS KNOWN AND UNKNOWN ABOUT COMMUNICATION BETWEEN NEURONS AND MICROGLIA

During the last decades, it has become evident that the functions traditionally ascribed to microglia, i.e. to clear dead cells and debris and to mediate brain inflammatory states, are only a limited fraction of a much wider spectrum of functions spanning from brain development to aging and neuropathology (Polazzi and Contestabile, 2002). Now it is well known that these cells are capable of integrating ad responding appropriately to different inputs *(*Hanisch, 2002; Schwartz et al., 2006; Hanisch and Kettenmann, 2007) and microglial activation is a process more diverse and dynamic than has been considered in the past.

Although ramified microglia in the CNS show a more quiescent immunological profile than other tissue macrophages (and how this is achieved is less understood) these cells are not anymore considered as 'resting' because 2-photon microscopy showed highly motile processes of these cells that actively screen their microenvironment (Davalos et al., 2005; Nimmerjahn et al., 2005; Haynes et al., 2006).

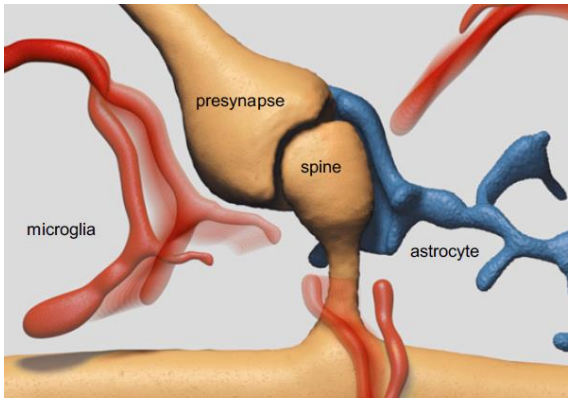

Fig.2. Dynamic interaction of microglial processes with the tripartite synapse (Kettenmann et al., 2013).

With more than 1 to 3 mm/min process extensions and retractions, microglial processes in the healthy brain make brief, repetitive contacts with synapses at a frequency of about once per hour. Wake and colleagues reported that microglial processes appear in a close proximity to presynaptic boutons, where they remain for about 5 min and then retract (Wake et al., 2009).

The brain is thus under permanent microglial surveillance (Fetler and Amigorena, 2005), which supports the earlier concept of microglia as the first line of defense in the CNS (Kreutzberg, 1996; van Rossum and Hanisch, 2004) and, once activated, microglia

specifically interact with neurons, influencing their survival either in a positive or in a negative direction.

Concerning neurons, so far, they mainly have been described as victims of (over)activated microglia, with limited influence on microglia functions and activity. Multiple evidence showed that neurons inform microglia about their status and are capable of controlling microglia function, thereby contributing to the inflammatory milieu of the CNS. Thus, neurons can be supposed as key immune modulators in the brain (Biber et al., 2007).

This highlights the functional interplay between these two cell types, with microglia controlling apoptosis and synaptic properties, and neurons being able to influence microglial activation. In general, the brain is the biggest puzzle for the immune system with a very thin line separating protection and restoration from toxicity and destruction (Turrin and Rivest, 2006).

1.2.1 Physiological conditions

Many factors, including chemokines or small molecules like ATP, are detectable by microglia in case of neuronal injury. Moreover microglial activation in the healthy brain is limited because once activated, microglial immune function is rapidly turned down to avoid the development of unwanted side effects, that may cause secondary neuronal damage (Galea et al., 2007). Microglia control neural networks either through removal of cellular and subcellular elements (by phagocytosis) or through secreting multiple factors with transmitter, trophic or neuroprotective properties. The variety of neuro-active agents that microglial cells can secrete has been mainly studied in cell culture.

Conceptually, two kinds of signals control microglial behavior in response to injury: ‘‘find-me’’ signals attract microglial cells in proximity of the damaged site, whereas ‘‘eat-me’’ signals allow microglia to identify the target and trigger phagocytosis. According to another classification microglial immune function is controlled by two classes of signals, named ‘Off’ and ‘On’ signals (Biber et al., 2007).

The former are constitutively found in the healthy, normal functioning brain microenvironment, thus the disappearance of Off signals *per se* causes microglia responses.

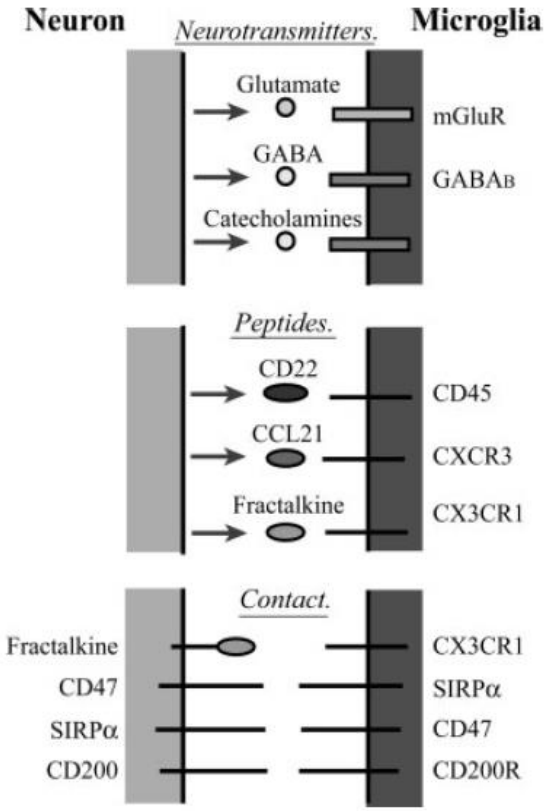

Fig.3. Molecular actors of neuron to microglia communication. Information flow from neuron to microglia is achieved through contact or by secreted mediators (Bessis et al., 2007).

By contrast, On signals act and operate on demand to initiate a defined microglial activation program (pro- or anti-inflammatory) (Biber et al., 2007).

On the other hand, for the control of microglia, neurons utilize different classes of signaling molecules, such as purines, neurotransmitters or membrane bound molecules that may function as “On” or “Off” signals (Biber et al., 2007; Hanisch and Kettenmann, 2007; Pocock and Kettenmann, 2007).

Studies *in vitro* demonstrated that microglial cells express a variety of receptors for neurotransmitters, neuropeptides, and neuromodulators, being able to sense neuronal activity (Pocock and Kettenmann, 2007). In general, in the normal brain the immune response of microglia is kept quiescent by neurons through both humoral and cell-cell contact mechanisms.

1.2.1a Neurotransmitters

Cultured microglial cells express adrenergic receptors, metabotropic and ionotropic glutamate and g-aminobutyric acid (GABA) receptors, dopamine receptors, bradykinin receptors, and several types of purinoceptors, which influence their electrophysiological membrane properties (Farber and Kettenmann, 2005; Inoue, 2006).

For example partial activation of both GABAA and GABAB receptors renders microglia less responsive to inflammatory stimuli such as lipopolysaccharide (LPS) and interferon-gamma (Lee et al., 2011). Similarly, glycine, which is the other main inhibitory neurotransmitter, also attenuates the production of inflammatory cytokines and the phagocytic activity of brain macrophages (Schilling and Eder, 2004) and noradrenalin reduces the LPS-stimulated release of NO, IL-6 and TNFa. Dopamine might also regulate microglial release, but this issue remains debated (Chang and Liu, 2000; Farber and Kettenmann, 2005).

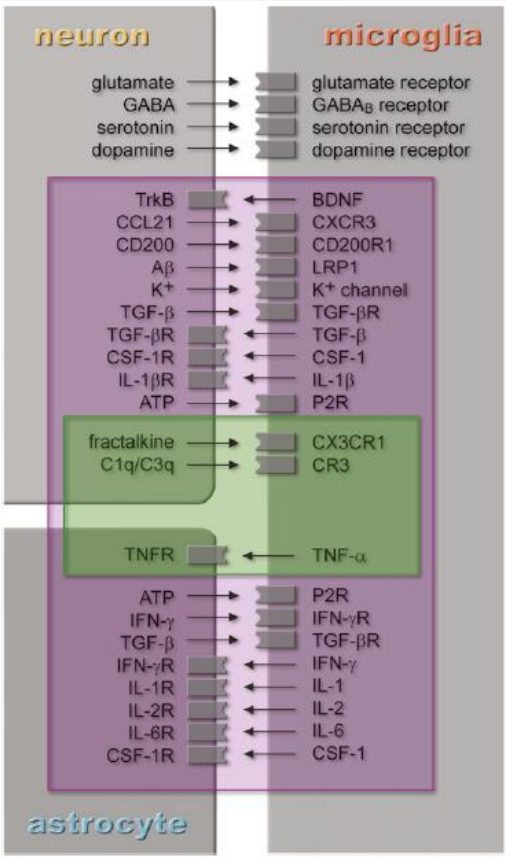

Fig.4. Neuron-microglia and astrocyte-microglia signaling pathways in pathology and physiology (Kettenmann et al., 2013).

Finally, stimulation of vagus nerve attenuates peripheral macrophage activation through the release of acetylcholine (Borovikova et al., 2000; Wang, H. et al., 2003). These studies suggest that neurotransmitters could in general reduce microglial released factors (Farber and Kettenmann, 2005). However, the role of glutamate, which is the main excitatory neurotransmitter, remains to be clarified.

These findings raise the hypothesis that microglia can sense neuronal activity based on local neurotransmitter levels and that this Off signaling limits microglial release of proinflammatory molecules.

It is now evident that neurotransmitters instruct microglia to perform distinct types of responses, from triggering an inflammatory cascade to acquiring a neuroprotective phenotype (Pocock and Kettenmann, 2007).

Although a diversity of transmitter receptors has been described on microglia, so far we lack experimental proof that these receptors are activated during synaptic transmission because their expression has only been studied in acute brain slices where the microglial activation process is already initiated. It is likely, however, that microglia in the healthy brain already express many (if not all) of such receptors (Kettenmann et al., 2013).

1.2.1b Peptides

Due to numerous observations that damaged neurons *in vivo* are rapidly surrounded by activated microglia, it is suggested that injured neurons emit microglia attractors (Streit et al., 1999; de Jong et al., 2005). Except for C chemokines, members of all other chemokine families have so far been described in neurons (de Haas et al., 2007) and interestingly, for most of them microglia express the corresponding receptors and respond *in vitro* with chemotaxis (Biber et al., 2001; Biber et al., 2002a; Biber et al., 2002b; Dijkstra et al., 2004; de Haas et al., 2007).

A valid example is CX3CL1 (CX3C chemokine ligand 1, also known as fractalkine) a membrane bound protein that can be also released from the cell by cleavage from the surface by proteases of the A Disintegrin and Metalloprotease (ADAM) family (Garton et al., 2001; Hundhausen et al., 2003). Since its receptor (CX3CR1) is expressed only by microglia in the CNS (Verge et al., 2004), a role of CX3CL1–CX3CR1 signaling in neuron–microglia interaction has been suggested (Harrison et al., 1998).

However, it is yet not clear whether membrane bound or soluble CX3CL1 is responsible for microglia inhibition *in vivo*. *In vitro*, it has been shown that neurons release CX3CL1(Erichsen et al., 2003; Limatola et al., 2005). Moreover, Cardona and colleagues reported soluble CX3CL1 concentrations up to 300 pg/ml in aqueous extracts derived from non-inflamed brain, which suggests that CX3CL1 is constitutively released in the normal CNS.

The same authors showed that loss of CX3CR1 function increases microglial neurotoxicity upon central nervous system alteration (Cardona et al., 2006) supporting the idea that neuronal fractalkine restrains microglial function. The existence of a neuronal membrane-anchored fractalkine with a microglial receptor suggests that neuron-microglia crosstalk also takes place by contact.

Furthermore, transforming growth factor-beta (TGF-b) is an anti-inflammatory cytokine that is also constitutively expressed in neurons. Because increased microglia activity was observed in brains of TGF-b-deficient mice (Brionne et al., 2003), TGF-b might contribute to Off signaling in the non-inflamed brain. However, it is unclear whether the brain inflammation in TGF-b-deficient mice is a direct effect of microglia activity or a consequence of neurodevelopment disturbances due to the lack of TGF-b growth promotion (Biber et al., 2007).

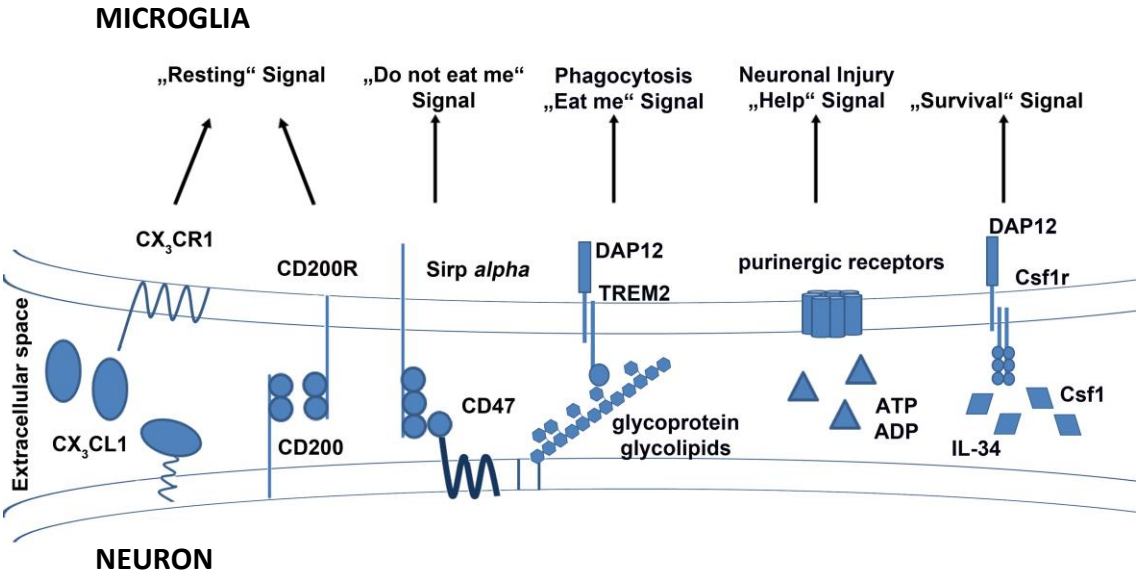

Fig.5. Exogenous signals and their receptors on microglia (Kierdorf and Prinz, 2013).

1.2.1c Contact

Accumulating evidence shows that neurons express several immunoglobulin superfamily (IgSF) molecules on their cell membrane that potentially suppress microglial immune function and microglia have to be in close regular contact with neurons to ensure IgSF signaling (Biber et al., 2007).

The best characterized molecules of this superfamily in the CNS are CD200 (in the rat formerly known as OX-42) and CD47, both of which are found to be constitutively expressed at the neuronal membrane surface (Webb and Barclay, 1984; Hoek et al., 2000; Wright et al., 2001). The transmembrane receptor for CD200 (CD200R) in the brain is primarily present on microglia. Studies in CD200-deficient mice corroborated this finding because the animals displayed worsened disease outcome and enhanced microglia activity

in Experimental Allergic Encephalomyelitis (EAE), as well as in facial nerve transection, retinal inflammation and encephalitis (Hoek et al., 2000; Broderick et al., 2002; Deckert et al., 2006). Moreover, being cells of myeloid origin, microglia express the leukocyte common antigen CD45 at low levels and it has been shown *in vitro* that CD45 activation leads to inhibition of microglia activity (Tan et al., 2000; Biber et al., 2007). In mixed neurons/microglia cultures, neurons secrete CD22 which inhibits proinflammatory cytokines production by microglia through binding to the transmembrane tyrosine phosphatase CD45 (Mott et al., 2004). A similar function could be postulated for neuronal CD47 and its receptor CD172a (also known as SIRPa or SHPS-1) (Ohnishi et al., 2005).

1.2.2 Pathological conditions

Damaged neurons are surrounded by activated microglia *in vivo* within hours after injury, which suggests that neurons emit On signals that attract these cells and can initiate protective or detrimental microglia function. When neuronal cells die, independently of the specific cause of death, the reaction of microglia is stereotyped and consists of quick transformation into activated microglia (Spranger and Fontana, 1996). In general microglial activation after neuronal injury primarily reflects a protective effort and microglial neurotoxicity can occur after excessive and uncontrolled stimulation of microglia (van Rossum and Hanisch, 2004; Cardona et al., 2006) or when microglia function is impaired (Boillee et al., 2006; Streit, 2006; Neumann and Takahashi, 2007).

Several factors are considered to be pathological signals secreted or leaked by damaged or overactive neurons, they include multiple types of cytokines (e.g., TNF-a or ligands for receptors such as CCR1, 3, 5, and 7 and CXCR1 or 3), trophic factors like brain derived neurotrophic factor (BDNF), the gaseous transmitter NO or neurotransmitters (ATP and glutamate)(Kettenmann et al., 2011).

Chemokine-filled vesicles have been found in neurons in the soma, in axons and at pre-synaptic sites (de Jong et al., 2005; Rostene et al., 2007; Jung et al., 2008). Thus, although it has yet not been conclusively shown that endangered neurons transport and release chemokines to the site of microglia activity, these data strongly corroborate this hypothesis.

The fact that most of microglia-derived factors are considered to be detrimental may reflect global upregulation in response to pathology. In physiological contexts the upregulation of some factors may be simply confined to distinct sites within the microglial cell (at sites where they interact with neuronal structures) or limited to a small population

of microglia that is engaged in a defined interaction with neurons (Kettenmann et al., 2013).

In the *in vivo* experiments by Davalos and colleagues it is clear that lowering extracellular ATP concentration by the ATP-hydrolyzing enzyme apyrase results in reduced process movements, whereas artificially created ATP gradients stimulate their motility (Davalos et al., 2005). The metabotropic P2Y12 purinoceptors are responsible for that type of movement control (Haynes et al., 2006) and surprisingly, the expression of P2Y12 receptors is mainly found in ramified cells and is downregulated by microglia activation, indicating that P2Y12 senses purine release at an early phase after neuronal injury.

After neuronal damage, other microglial purine receptors are upregulated *in vivo*, as has been demonstrated for P2Y6. This receptor doesn't contribute to microglial morphological changes but triggers microglial phagocytosis (Koizumi et al., 2007). In general, microglial purine receptor expression pattern can change rapidly, with profound impact on microglia function (Kettenmann, 2007).

Even though ATP and purinoceptors are good potential candidates, the molecules that attract microglial processes to the synapses remain largely unknown.

In addition, microglial TNF-a and microglial ATP act in concert to stimulate astroglial ATP release which subsequently amplifies the microglial signal and promotes astroglial release of glutamate that directly affects synaptic transmission through presynaptic metabotropic glutamate receptors (Pascual et al., 2012).

Excessive neuronal glutamate release is liked to neurodegenerative processes. Glutamate not only directly leads to neuronal death, but also serves as an activation signal for microglia.

In culture, microglia express several glutamate receptors (GluRs), such as AMPA-type GluR1–GluR4, kainate receptors, and members of all three groups of metabotropic glutamate receptors (mGluRs) (Taylor et al., 2002; Taylor et al., 2003; Hagino et al., 2004). Activation of various glutamate receptors induces release of TNF-a, which in sinergy with microglial-derived Fas ligand leads to neurotoxicity (Taylor et al., 2005). Moreover neurons damaged by glutamate excitotoxycity rapidly express CCL21 that is packaged into vesicles, transported to presynaptic structures, and released through exocytosis (de Jong et al., 2005). Microglial cells express CXCR3 receptors specific for CCL21 and microglial stimulation with CCL21 triggers chemotaxis and increases migratory activity (Biber et al., 2001).

Moreover also the matrix metallo protease 3 (MMP-3) is released in its active form from apoptotic neuronal cell lines and apoptotic mesencephalic neuronal cultures (Kim et al., 2005; Kim et al., 2007). It has been shown that the catalytic domain of recombinant MMP- 3 (cMMP-3) mediates the release of TNF-a, IL-6, IL-1b and IL-1ra (IL-1 receptor antagonist) into the supernatant of microglia cultures. Thus, the release ofMMP-3 can be considered a direct neuroglial apoptosis signal in neurodegeneration (Schwartz et al., 2006).

Finally microglia have a unique pattern of potassium channels, so they are able to respond quickly to neuronal depolarization (Eder, 1998) and increased levels of extracellular potassium around injured neurons may induce microglial activation as (Gehrmann et al., 1993).

1.3 RECENT EVIDENCE ON MICROGLIAL SENESCENCE

In contrast to acute CNS injuries, neurodegeneration occurring in long-lasting diseases such as Alzheimer's disease (AD) and in Parkinson's disease (PD) is a chronic process that usually takes decades to develop. Recent studies give more support to the hypothesis of microglia senescence in the aged brain, which offers a novel perspective on aging-related neurodegeneration. In particular the hypothesis claims that microglial senescence, by rendering microglia function abnormal and unable to respond correctly to stimuli, is a key factor contributing to progressive neurodegeneration and its associated neuronal cell death. In general, it seems that the physiological neuroprotective function of microglia that characterizes the young brain is progressively lost with aging (Luo et al., 2010).

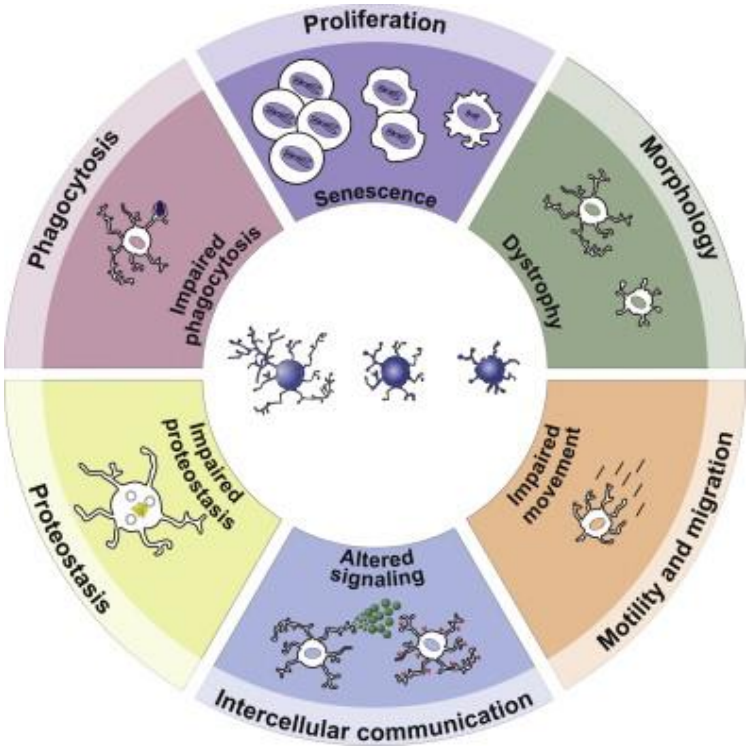

Fig.6. The hallmarks of microglial aging.
The six major effector functions/phenotypes of microglia are highlighted at the outer ring of the scheme. Within the inner ring of the circle are described the effects of aging on each of these functions (Mosher and Wyss-Coray, 2014).

Even though this novel hypothesis provides a potential therapeutic target of improving microglia function by delaying microglia senescence, before this proposal can be fully accepted, there are still some important questions that need to be answered. For example, a significant question is: what is the difference between "activated microglia" and "aged microglia"? (Luo et al., 2010).

Streit et al. described that "hypertrophy and retraction and thickening of process" characterize activation while "deramification, shortening and twisting of processes, cytoplasmic fragmentation" are features of aging (Streit et al., 2008). However, so far no definite biomarker or morphology feature can clearly discriminate these two stages.

Nowadays the best way to discriminate senescence versus activation is to determine microglial response to injury and whether they ultimately return to ramified resting state after stimuli and/or whether the changes occur in a physiological environment free of pathologic stimuli (Luo et al., 2010).

In general, in the brain of older human subjects dystrophic microglia are prevalent and extensively distributed (Streit et al., 2004; Wasserman et al., 2008), whereas normal ramified microglial morphology with only rare cases of dystrophic microglia are seen in the young brain (Conde and Streit, 2006).

In addition to dystrophic morphology, telomere shortening has also been demonstrated for glial cells in the aged brain. It is well known that senescence is triggered once any cell telomere, the physical ends of eukaryotic chromosomes, reaches a critically-short length (Hemann et al., 2001) due to the inability of DNA polymerase to completely replicate linear DNA molecules. Therefore, with a limited replicated potential, cells change remarkably their function and gene expression.

Concerning microglial senescence Flanary et al. reported a direct evidence in support of microglial telomere shortening and reduction of telomerase activity during normal aging in rats and of a tendency in humans toward telomere shortening with presence of dementia. In particular, human brains containing high amyloid loads demonstrate a significantly higher level of microglial dystrophy than nondemented, amyloid-free control subjects. For this reason the authors hypothesized that microglia in AD individuals may be less able, or totally unable, to maintain neuronal health and effectively phagocyte amyloid plaques and they suggest that microglial degeneration is an important factor in the pathogenesis of AD (Flanary et al., 2007).

In particular, as the only mature cell of the CNS with an appreciable mitotic ability, it is likely that the microglial replicative potential become exhausted with aging. The decline in microglial renewal capacity may be further diminished by, and contribute to, the presence of amyloid, which consequently may accelerate the process of telomere shortening (Flanary et al., 2007).

Interestingly, in the aged brain, microglia are not universally senescent since scattered dystrophic microglia are usually found nearby normal ramified microglial cells, suggesting that only a subset of microglia become dystrophic, and not all of them are of the same age and functional state (Streit and Graeber, 1993).

It is possible that the extent of this subset progressively increases with aging and ultimately exceed the neuroprotective normally-functioning microglial cells. These age-associated

changes may underlie the alterations of microglial function and their distinct responses to injury (Luo et al., 2010).

However, there are still many other questions that remain difficult to answer so far: is microglial activation in the aging brain co-existing with microglial dystrophy or is it a consequence? Considering microglia as physiologically multi-functional cells, which specific function(s) is /are primarily altered by microglia dystrophy? Is there a particular microglial function predominantly related to neurodegeneration? It is evident that more specific research is required to answer these and many more questions (Luo et al., 2010).

1.4 CULTURING MICROGLIA: CRITICAL ASPECTS AND COMMON MISCONCEPTION

The rebirth of microglia in the 1980s produced a major misconception regarding functional roles of activated microglia, i.e. that microglial activation is harmful to neurons in the CNS. This concept emerged mostly when researchers began to culture microglial cells.

The pioneering work done by Giulian and Baker, established a relatively simple procedure for isolating microglia from the cortex of a rat (or mouse) before (or early after) birth and for maintaining the cells in culture (Giulian and Baker, 1986). Using a specific process of adhesion and shaking of cultured glial cells, a purified culture of approximately 95% enriched microglia can be obtained. Their method, although sometime slightly manipulated, is still the procedure of choice for exploring several aspects of microglia biology (Streit, 2010).

But we have to take into account that the removal of these cells from their micro-environment releases them from the normal constraint that plays an important and extremely critical role in their phenotype and that neonatal microglia have not experienced the CNS milieu *in vivo* in the context of an intact, mature blood-brain barrier (BBB) (Ransohoff and Perry, 2009).

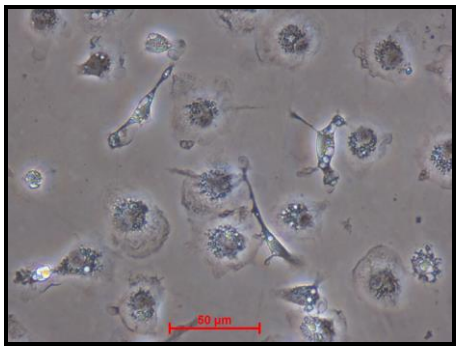

Fig.7. Representative culture of primary microglial cells harvested from newborn rats.

Most importantly, the generation of microglial cells cultures involves extreme brain damage (chopping and trituration of dissected tissue) inevitably causing immediate

activation of microglia and their transformation into brain macrophages (Streit et al., 1999).

Furthermore years of studies were focused on the additionally stimulation of microglia with LPS and/or interferon-gamma, resulting in super-activated cells that produce several secretory products. A first misconception started with the silent assumption that cultured microglia unstimulated by LPS were representative of resting microglia in the normal brain, and the consideration that LPS-activated cells were analogous to activated microglia in the damaged brain.

Actually, cultured microglia, before LPS stimulation, are already at an activation state that is equivalent to what is perceived as maximal microglial activation *in vivo*, that is, the brain macrophage stage. Therefore, it is important not to equate microglial activation *in vitro* with microglial activation *in vivo* (Streit, 2010).

Moreover microglia activated *in vitro* with LPS or other immune-stimulants can produce potentially neurotoxic molecules, such as nitric oxide, glutamate, reactive oxygen and nitrogen species, and proinflammatory cytokines. These observations have been extrapolated to mean that activated microglia *in vivo* are harmful and could be responsible for exacerbating damage in the injured or diseased CNS by producing neurotoxic compounds that cause neurodegeneration secondarily.

On the same direction went McGeer's identification of activated microglia expressing major histocompatibility complex antigens in the brains of humans AD patients (McGeer et al., 1987). His assumption was not entirely correct because MHCII expression occurs prominently also on non-activated microglia and perivascular cells in the normal human and animal brain (Streit, 2010).

Moreover there is a good chance that non-activated (especially dystrophic) microglia were misidentified as activated cells based not only on the false assumption that MHCII expression is an immunological marker for activated cells, but also because of only a superficial assessment of their morphology. In the late 1980s and early 1990s the idea of morphologically abnormal (dystrophic) microglia did not exist and researchers were quick to identify any non-ramified microglial cells as activated. However, conclusions from cell culture studies are difficult to reconcile with *in vivo* observations that show that microglial activation is the result of neural tissue damage rather than its cause, underscoring the basic concept of inflammation, namely, that inflammation is the cellular response to tissue injury (Streit, 2010).

Considering AD, a large number of other inflammatory mediators, including many cytokines, were being added to the list of substances thought to be consistently increased in the affected brain. Furthermore due to this neuroinflammation hypothesis of AD pathogenesis, numerous clinical trials with anti-inflammatory drugs have been developed, none of which have shown a clear benefits for slowing or preventing disease onset and progression (Streit, 2010).

A potential problem with all these studies, focused on measuring various inflammatory molecules in the AD brain, is that most of the inflammatory proteins (similar to MHC antigens) are also expressed in non-AD brains, and their levels are likely to change substantially depending on whether or not peripheral infections are present. In fact even though it is clear that peripheral infections in humans are associated with prominent microglial activation, studies that have assessed neuroinflammation in AD did not make any distinction between AD cases with and without infectious disease, which are quite common in AD patients (Streit, 2010).

In conclusion, elucidation of many aspects of microglia can be clearly investigated *in vitro*, but before accepting them, the direct relevance of these observations needs to be carefully established *in vivo*. Even though the state of cells grown on glass or tissue culture plastic is likely more close to inflammatory cells rather than to steady-state microglia and the extensive preparation procedures needed for each experiment makes this model more time consuming compared to other microglia cell lines, for sure primary cultures maintain similar cell properties and they are still the best tool for studying microglial cells *in vitro* (Stansley et al., 2012).

1.5 RAT CEREBELLAR GRANULE NEURONS AND THEIR APPLICATION TO IDENTIFY NEUROPROTECTIVE MOLECULES

Neurons, like many other cell types, require survival factors to inhibit the apoptotic machinery and prevent death. Apoptosis is observed not only in neurodegenerative diseases but also during the physiological development of the nervous system (Facci and Skaper, 2012).

To answer many experimental questions, an appropriate *in vitro* cell-culture system with particular characteristics is required. Among the available neuronal cell lines, several are not suitable due to changes in their phenotype when compared to cells from which their originate. Primary culture, alternatively, can serve as an appropriate model of highly differentiated neurons. However, a drawback of using primary cells is that most of the cultures consist of mixed neuronal populations. Also, in particular circumstances, the presence of non-neuronal cells (mainly astrocytes) may be a problem, as they have different functional and metabolic characteristics compared to neurons. As a compromise, cerebellar granule neurons are often used (Kramer and Minichiello, 2010).

Granule cells of the cerebellum constitute the largest homogeneous neuronal population of mammalian brain, located in the deepest layer of the trilaminar cerebellar cortex. Due to their postnatal generation and their well characterized use as primary *in vitro* cultures, cerebellar granule cells are a model of election for the study of cellular and molecular mechanisms of survival/apoptosis and neurodegeneration/neuroprotection (Contestabile, 2002).

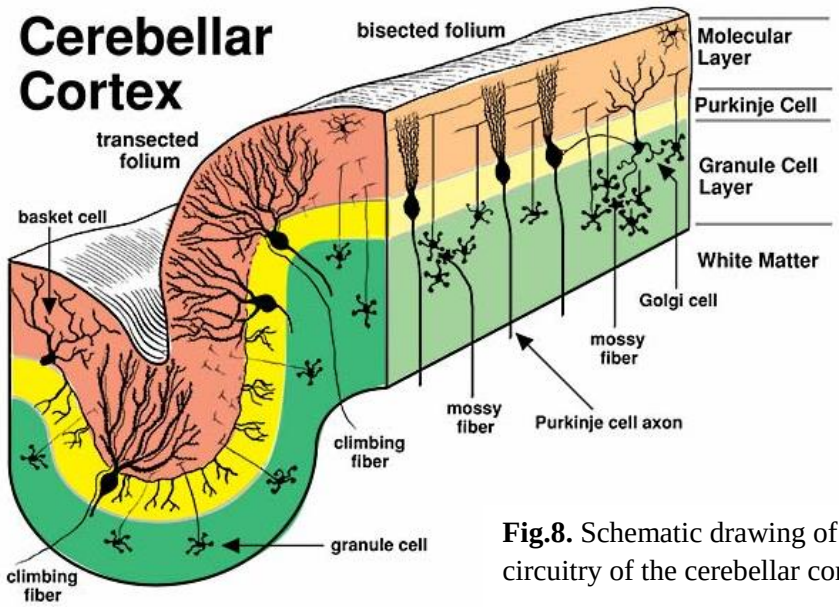

Fig.8. Schematic drawing of the cytology and circuitry of the cerebellar cortex.

In primary dissociated cultures of early postnatal cerebellum, granule cells are highly enriched, 95% of the cells in culture are cerebellar granule neurons, and maintain many properties of developing granule neurons *in vivo* (Lasher and Zagon, 1972; Gallo et al., 1987). Moreover they mature very late (postnatal) in the development of the central nervous system. These features allow cerebellar granule cells to be isolated from newly born animals, 7 days post-natal, and to be cultured *in vitro* (Kramer and Minichiello, 2010).

Importantly, as for all neuronal cultures, also in this model the circuitry of the brain and cells connections are lost. The input that CGN receive from mossy fibers and the connection they form with Purkinje neurons *in vivo*, are disrupted in an *in vitro* system. To overcome this problem, synaptic activity is mimicked by chronic depolarizing conditions usually obtained by exogenously raising potassium concentration to > 20mM.

The resulting depolarization of cell membranes is thought to activate voltage-gated calcium channels, thus increasing cytoplasmic calcium levels that consequently leads to an activation of gene transcription that prevents cell death (Kramer and Minichiello, 2010). The apoptotic process consequent to the shift of mature granule neurons (after 7 days *in vitro*) to low potassium concentration, which is usually accompanied by serum deprivation, occurs in a relatively short time (neuronal death reaching 30%-50% after 16-24 hours), is highly reproducible and is, therefore, very useful for studies aimed at the identification of cellular signals activated in apoptotic neurons as well as for testing ways of protecting them from apoptotic death (Contestabile, 2002). Removal of CGN from depolarizing conditions with high K+ has found widespread application as a model for studying neuroprotective molecules and their mechanisms of action (Facci and Skaper, 2012).

Even though not described in the experiments of this thesis, the inhibition of the proteasome has also been shown to induce apoptosis in cultures of cerebellar granule neurons, in parallel with the activation of caspase 3 (Contestabile, 2002).

The use of the toxin 6-OHDA has been adopted extensively in the present work. In particular, 6-OHDA is a neurotoxin with selectivity for dopaminergic neurons because of the presence of the dopamine transporter expressed in these cells, but CGNs are quite sensitive to 6-OHDA too (Dodel et al. 1999; Lin et al. 2003; Chen et al. 2004; Ma et al. 2006; Monti et al. 2007). In CGNs, 6-OHDA results in neurodegeneration presenting most of the characteristic features of Parkinson-like neuronal death, i.e. an apoptotic process with oxidative stress, proteasome impairment and protein aggregation (Polazzi et al. 2009).

Another apoptotic stimulus used in my experiments is glutamate. In fact it is well known that in cultured rat cerebellar granule cells, glutamate or N-methyl-D-aspartate (NMDA) activation of the NMDA receptor caused a sustained increase in cytosolic Ca_2 levels ($[Ca_2]i$), reactive oxygen species (ROS) generation, and cell death but no increase in caspase-3 activity (Li et al., 2004).

As a concluding remark it is clear that cerebellar granule neurons, being one of the most reliable models for the study of neural development, function and pathology, have acquired an important position in modern neuroscience (Contestabile, 2002).

1.6 NEUROPROTECTIVE FACTORS SECRETED BY MICROGLIAL CELLS

Microglia are well known for their involvement in rescuing injured neurons in *in vivo* models of neurodegeneration and the same beneficial activation-dependent neuroprotection has also been observed in several *in vitro* co-cultures studies: microglia-derived neuroprotective factors can protect metabolically impaired neurons (Park et al., 2001) as well as prevent nitric oxide-induced apoptosis of cortical neurons in a co-culture model (Toku et al., 1998).

Furthermore, Microglia Conditioned Medium (MCM) is able to promote survival and development of cultured mesencephalic neurons harvested from embryonic rat brain (Nagata et al., 1993) and, in addition, plasminogen (PGn), which was identified as a microglial secretory product, increases dopamine uptake in cultured rat dopaminergic neurons (Nakajima et al., 1994).

A study based on microglia-embryonic dopaminergic neurons co-cultures reported that MCM decreased the survival of dopaminergic neurons in primary cultures and, on the contrary, the direct contact of microglia with the same neuronal cells shifted the effect from toxic to survival-promoting (Zietlow et al., 1999).

In our lab it was previously demonstrated that unstimulated *in vitro* microglial cells physiologically release in the medium molecules able to rescue neurons in different models of induced apoptosis. Moreover, diffusible signals from apoptotic neurons enhanced these microglial neuroprotective properties (Polazzi et al., 2001; Eleuteri et al., 2008). More recently, we extended our studies to an *in vitro* model of Parkinson-like neuronal death using the toxin 6-hydroxydopamine (Polazzi et al., 2009). The group demonstrated that the microglial neuroprotective property was exerted through the release of peptidic molecules, which cooperate with low molecular weight, heat-resistant factor(s), in neuroprotection. In

particular, transforming grow factor-β2 (TGF-β2) has been identified as one of these neuroprotective agents (Polazzi et al., 2009).

A neuroprotective effect of microglia was also observed in a model of culture of neurons exposed to aggregated Abeta 1-40, through the release of apolipoprotein E (ApoE) in the culture medium.

In this study the immunodepletion of ApoE or targeted inactivation of the ApoE gene in microglia abrogated neuroprotection by MCM, whereas supplementation with human ApoE isoforms restored protection, which, interestingly was potentiated by the presence of microglia-derived cofactors (Qin et al., 2006). A similar beneficial microglial activation-dependent neuroprotection has also been demonstrated in another model of *in vitro* neurotoxicity, i.e. oxygen glucose deprivation (OGD) in hippocampal organotypic cultures, where microglia protected against the neuronal damage and formed a close physical cell–cell contact with neurons in the damaged slice area (Bahr, 1995; Neumann et al., 2006).

In addition, in a co-culture system composed of BV-2 microglia line overexpressing the macrophage colony-stimulating factor receptor (M-CSFR) and hippocampal organotypic slices treated with NMDA, the over-activation of microglial M-CSFR, through endogenous neuronal M-CSF was responsible for microglial neuroprotection against NMDA excitotoxycity (Mitrasinovic et al., 2005).

These data strongly suggest that microglial cells not only minimize the dangerous consequences of neuronal damage through the elimination of apoptotic cells, but also give functional support to the remaining neurons. As a conclusive remark, even with all the limitations that *in vitro* systems may have, these models allow to identify neuroprotective factors secreted by microglia and, in turn, to discover neuronal or astrocytic molecules able to modulate their production. These studies are relevant to stimulate further therapeutic approaches aimed at the treatment of neurological disorders (Polazzi and Monti, 2010).

1.6.1 Proteomic analysis of microglial conditioned medium: identification of SOD1 and ApoE

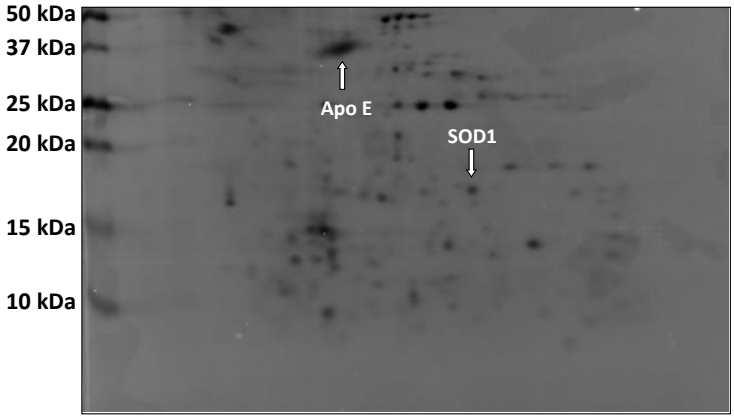

Fig.9 Identification of SOD1 and ApoE release and accumulation in the MCM. A representative Coomassie Blue-stained 2-D gel is illustrated and the arrow indicates the position of the spots identified through mass spectroscopy analysis as SOD1 and ApoE. Modified from Polazzi, Mengoni et al. 2013.

Thanks to a proteomic analysis we could identify many proteins present in the medium conditioned by microglial cells for 48h, whose neuroprotective properties have been well documented in previous studies (Polazzi et al., 2001; Polazzi et al., 2009). This analysis demonstrated the presence of several spots, revealing the accumulation of various peptides in the conditioned medium. As highlighted in figure 9, we decided to focus our attention on two particular proteins: SOD1 and ApoE, chosen for different reasons. SOD1 is mainly localized in the cytosol, so we have been attracted by the fact that it could be secreted by microglial cells, while the choice of ApoE was due to the fact that its spot, compared to others, was very intense.

1.7 SUPEROXIDE DISMUTASE 1

Superoxide anions, oxygen molecules with an extra electron, are produced as a result of cell metabolism, especially oxidative phosphorylation. If not scavenged they can cause damage to cell membranes, DNA and cellular organelles.

Copper, zinc-superoxide dismutase-1 (SOD1), is a member of a group of isoenzymes involved in the scavenging of superoxide anions. The human SOD family of proteins also includes manganese superoxide dismutase (SOD2) and the extracellular superoxide dismutase (SOD3).

Even though they similarly function as anti-oxidizing enzymes, they differ for structure, chromosomal location, cofactors, genic distribution and cellular localization (Parge et al., 1992). SOD1 constitutes ~1% of total protein in the cell (Pardo et al., 1995), and resides mainly in the cytosol with some degree of localization in the mitochondrial inner membrane space (Fukai and Ushio-Fukai, 2011).

SOD1 is a homodimer of 32 kDa with a Cu and Zn binding site, one of each per subunit.

Each monomer has a β-barrel structure and has two functional loops, where the metal binding regions are located: the electrostatic loop, that guides superoxide into the Cu^{2+} redox active site, and the zinc-binding loop (Valentine et al., 2005).

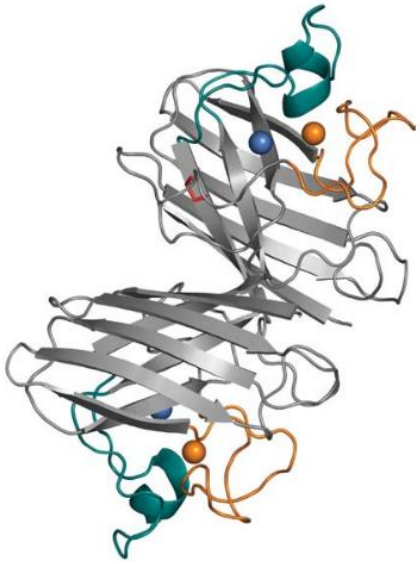

Fig.10. Crystal structure of metal bound dimeric human SOD1. Copper and zinc ions are shown as blue and orange spheres, respectively. The zinc loop is represented in orange and the electrostatic loop in green. The intra-subunit disulfide bond is shown in red. (Valentine et al., 2005)

In the enzymatic active site, SOD1 catalyzes the dismutation of superoxide to give dioxygen and hydrogen peroxide in a two-step process: one molecule of superoxide first reduces the cupric ion to form dioxygen and then a second molecule of O_2^- reoxidizes the cuprous ion to form hydrogen peroxide (Valentine et al., 2005).

The coordination of copper to SOD1 is required for dismutation of O_2^-, whereas other post-translational modifications, such as Zn^{2+} coordination (Kayatekin et al., 2008) and disulfide oxidation, help create a mature and structurally stable protein (Rotunno and Bosco, 2013).

Another functional feature of SOD1 is the presence of an intra-subunit disulfide bond between Cys57 and Cys146 (C57–C146). Both copper coordination and formation of C57-C146 is facilitated by the cytosolic copper carrier protein CCS (copper chaperone for SOD1) (Furukawa et al., 2004; Seetharaman et al., 2009).

Thus demetallation of SOD1 and/or reduction of C57–C146 destabilizes the protein and decreases the melting temperature (Forman and Fridovich, 1973; Furukawa and O'Halloran, 2005). These post-translational modifications are compromised in the context of diseases (Rotunno and Bosco, 2013).

1.7.1 SOD1 and disease

The enzyme superoxide dismutase (SOD) is a constitutive enzyme coded by a gene located in Chromosome 21 (21q22.1). Even though the tissues from patients with trisomy 21 contain 50% more SOD activity, the overexpression of SOD is unrelated to the symptoms observed in Down's syndrome patients. It has been proposed, that the increased SOD may be, instead, responsible for the increased incidence of Down's syndrome in children of older women. Interestingly, the augmented antioxidant protection resulting from an extra copy of chromosome 21 may, with time, selectively protect human oocytes from apoptosis, increasing their proportion with age, explaining the higher incidence of this disease (Turrens, 2001).

Growing scientific interest started in 1993, when mutations in the *SOD1* gene were reported to be involved in Amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disorder characterized by premature loss of motoneurons (Rosen et al., 1993). Because of the high incidence of *SOD1* mutations, which account for 20–25% of familial, or inherited, forms of ALS (FALS) cases, *SOD1* has been one of the most intensely studied genes in the ALS field and continues to be a primary therapeutic target (Bosco and Landers, 2010).

Importantly, motor neurons are the primary target in ALS, but ALS may actually represent a non-cell autonomous disorder for which glia play an active role (Ilieva et al., 2009). However, despite two decades of research on FALS-SOD1 *in vitro* and *in vivo*, the

exact mechanism of SOD1 in ALS pathogenesis remains unknown (Pasinelli et al., 2004; Ling et al., 2013).

Proteinaceous inclusions rich in mutant SOD1 have been found in tissues from ALS patients, ALS-SOD1 transgenic mice, and in cell culture model systems (Bruijn et al., 2004), leading many investigators to the conclusion that SOD1-associated fALS is a protein conformational disorder, similar to Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases in which protein aggregates are found (Soto, 2003; Valentine and Hart, 2003). The visible inclusions in SOD1-linked fALS contain neurofilament proteins, ubiquitin, and a variety of other components in addition to SOD1, but it is not known if copper, zinc, or any other metal ions are present in the inclusions or are involved in their formation. Nor is it known if the SOD1 polypeptide has been fragmented or otherwise covalently modified in the processes leading to aggregate formation (Valentine et al., 2005).

To date, at least 105 different mutations in the sod1 gene have been linked to fALS (Cleveland and Rothstein, 2001). The majority of these mutations cause amino acid substitutions at one of at least 64 different locations, but some cause frameshifts, truncations, deletions, or insertions (Cleveland and Rothstein, 2001; Andersen et al., 2003). (Most known fALS mutations are listed at http://www.alsod.org.)

Indeed, recent biophysical studies of fALS mutant SOD1 proteins suggest that the division into two groups termed metal-binding region (MBR) and wild-type-like (WTL) fALS mutant SOD1 proteins, on the basis of their SOD activities and metal-binding properties (Table 1) (Hayward et al., 2002; Rodriguez et al., 2002; Potter and Valentine, 2003). In particular the MBR subset of SOD1 proteins have mutations that are localized in and around the metal-binding sites, including the electrostatic and zinc loops, and were found to have significantly altered biophysical properties relative to wild-type SOD1. By contrast, the WTL subset of SOD1 protein was found to be remarkably similar to wild-type SOD1 in most of their properties (Valentine et al., 2005).

Wild-type-like (WTL) mutants				Metal-binding region (MBR) mutants
A4V[a,b]	G72S[a]	G93V[b]	N139K	H46R[a,b]
V7E[b]	D76Y[a]	E100G[b]	L144F[b]	H48Q[a,b]
L8Q[b]	L84V[b]	E100K[b]	L144S[b]	H80R[b]
G37R[b]	N86S[b]	D101N[b]	A145T[b]	G85R[a,b]
L38V[a,b]	D90A[a]	D101G[b]	V148G[b]	D124V[a,b]
G41D[b]	G93A[a,b]	I113T[b]	I149T[b]	D125H[a,b]
G41S[a]	G93R[b]	R115G[b]		S134N[a,b]
H43R[b]	G93C[b]	E133Del[a]		C146R[b]

Table 1. Isolated fALS mutant SOD1 proteins. In yellow are highlighted the mutations present in the plasmid used in these thesis (Valentine et al., 2005).

In contrast to FALS, much less is known about the etiology of sporadic ALS (SALS), which accounts for 90% of ALS cases. In fact, SALS may arise from genetic as well as environmental and behavioral factors. Although FALS is inheritable and SALS is not, the fact that FALS and SALS are clinically indistinguishable raises the possibility that they do in fact emerge from a common source and/or involve similar toxicity factors (Rotunno and Bosco, 2013).

Recent evidence supports SOD1 as a toxic factor that is common to a subset of both FALS and SALS. This evidence is largely based on the observation that aberrant conformations of WT SOD1, induced by oxidation, demetallation and other altered post-translational modifications, cause WT SOD1 to acquire the same toxic functions that are observed for FALS-associated SOD1 variants (Ezzi et al., 2007; Bosco and Landers, 2010; Guareschi et al., 2012).

1.7.2 Role as a signaling molecule

The physiological relevance of SOD1 catalysis extends beyond oxidative stress protection, playing also an important role in signal transduction.

For example, H_2O_2 generated by SOD1 can reversibly and specifically react with proteins, generally by oxidizing Cys residues, thus altering the biochemical and functional properties of those proteins in a redox dependent manner (Georgiou, 2002).

A variety of signal transduction pathways are modulated by H_2O_2, including, but not limited to, gene expression, cell proliferation, differentiation and death (Rhee, 2006; Brown and Griendling, 2009).

A representative example are NADPH oxygenases (Nox), that function as upstream regulators of these signal transduction pathways through the production of $O_2^{\bullet-}$, which is either converted to H_2O_2 spontaneously or catalytically by SOD1.

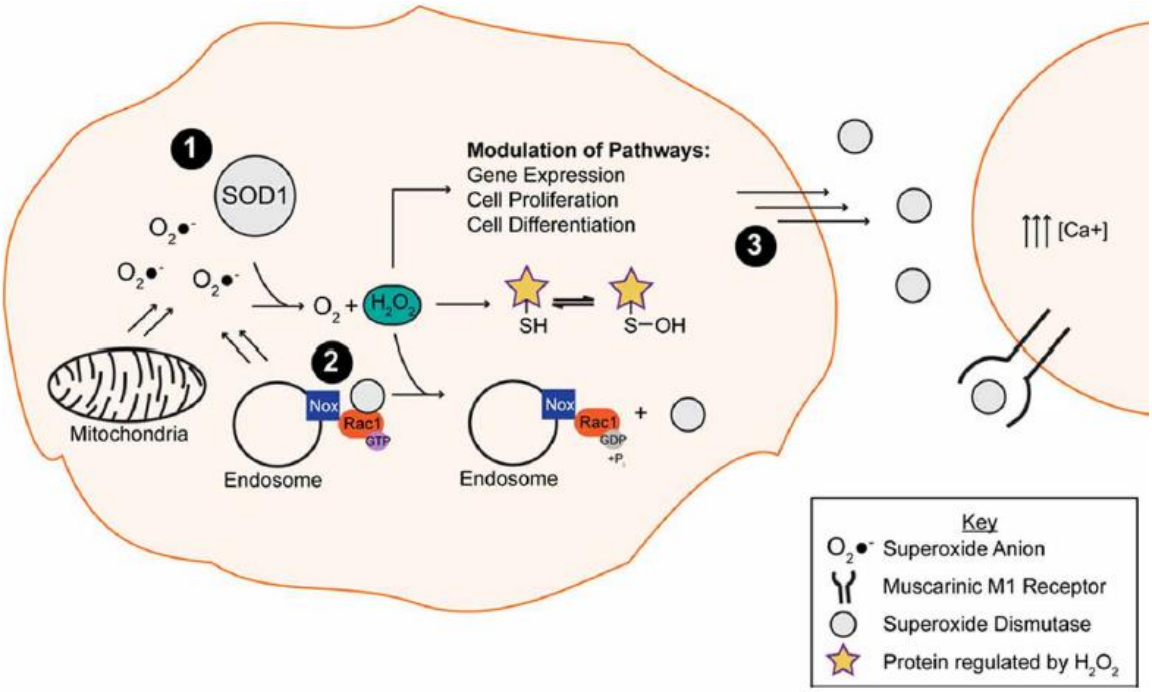

Fig. 11. The putative normal function of the native WT SOD1 protein. (1) SOD1 is responsible for converting the toxic superoxide anion ($O_2^{\bullet-}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). **(2)** SOD1 binds and stabilizes Rac1 in its active, GTP bound state, resulting in Nox2 (Nox) activation and superoxide production. The hydrogen peroxide by-product of the dismutase reaction of SOD1 and superoxide anion promotes the disassociation of SOD1 from the Rac1 complex, resulting in Nox inactivation. **(3)** The presence of extracellular SOD1 leads to an increase in intracellular calcium via a mechanism involving the phospholipase C/protein kinase C pathway (Rotunno and Bosco, 2013).

SOD1 comes into close proximity with Nox2-derived $O_2^{\bullet-}$ at the surface of endosomes in response to proinflammatory cytokines (Harraz et al., 2008).

A report by Harraz et al. (2008) demonstrated that SOD1 not only acts downstream of Nox2 but can also modulate Nox function through an interaction with Rac1.

SOD1 directly binds and stabilizes the active form of Rac1 in its GTP-bound state, leading to Nox2 activation and $O_2^{\bullet-}$ production.

Interestingly, H_2O_2 generated by SOD1 serves as a negative feedback of Nox2 activity: H_2O_2 induces the dissociation of the SOD1/Rac1 complex, thereby inactivating Rac1 and Nox2 (Harraz et al., 2008).

The mechanism for how H_2O_2 disrupts the interaction between SOD1 and Rac1 has not been elucidated. One possibility is that the H_2O_2 generated by SOD1, which is in close proximity to Rac1, oxidatively modifies Cys residues within Rac1 in such a way that disrupts the SOD1/Rac1 binding interaction (Rotunno and Bosco, 2013).
In this manner, SOD1 catalyzes a "molecular redox switch" that ultimately controls protein function and signaling, much like phosphorylation.

Extracellular SOD1 has also been shown to play a role in signaling. Although SOD1 is predominately localized to the cytoplasm, multiple reports have demonstrated that SOD1 is secreted in the form of microvesicles in both normal and transformed cells, including neuroblastoma SK-N-BE cells, through an ATP dependent mechanism (Mondola et al., 1996; Mondola et al., 1998; Cimini et al., 2002; Mondola et al., 2003; Turner et al., 2005).

The presence of extracellular SOD1 can activate the phospholipase C/protein kinase C pathway, implicated in calcium homeostasis, increasing intracellular calcium concentrations, mainly deriving from the intracellular calcium stores (Mondola et al., 2004). This effect is independent of SOD1 catalitic activity, and is totally inhibited by U73122, the PLC blocker, suggesting SOD1 as a neuromodulatory protein able to affect calcum-dependent cellular functions.
In particular, is has been suggested that SOD1 could activate a transductional pathway through the involvement of M1 muscarinic receptor, because the SOD1-dependent Ca^{2+} increase has a stimulatory effect on ERK 1/2 and Akt in SK-N-BE neuroblastoma cells. This effect was mimicked by the M1 agonist oxotremorine and prevented by the M1 antagonist pirenzepine and by M1 receptor knocking-down (Damiano et al., 2013). Of all other neurotransmitter systems, the muscarinic–cholinergic system carries out a key role in many important cerebral functions including learning, memory and synaptic plasticity (Scheiderer et al., 2006; McCoy and McMahon, 2007; Scheiderer et al., 2008).

Moreover, unlike the previous SOD1 signaling pathways discussed above, the activation of the M1 receptor does not appear to be dependent upon the production of $O_2^{\bullet -}$, as the ROS scavenger N-acetylcysteine did not alter the signaling effect of SOD1 in this context (Rotunno and Bosco, 2013).

1.8 APOLIPROTEIN E AND EVIDENCES IN FAVOR OF ITS NEUROPROTECTIVE ACTION

Apolipoprotein E (ApoE) is a 34kD glycosylated protein, originally identified for its involvement in cholesterol transport (Hatters et al., 2006). It also plays an important role in human neurological disorders, mainly Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA), as well as in acute brain injury by negatively affecting insult outcome (Friedman et al., 1999; Lynch et al., 2001; Holtzman et al., 2012).

In humans, three SNPs lead to changes in the coding sequence of ApoE and give rise to three common isoforms: ApoE2, ApoE3 and ApoE4. In particular, the presence of ApoE4 allele predicts an increased risk of developing late onset familial and sporadic AD, while ApoE2 lowers this risk (Hatters et al., 2006; Holtzman et al., 2012).

ApoE is the main apolipoprotein in the central nervous system (CNS) (Holtzman et al., 2012) and multiple functions have been ascribed to it: ApoE is involved in neurite outgrowth and neuronal plasticity (Korwek et al., 2009) and it regulates β-amyloid structure, clearance and neurotoxicity (Drouet et al., 2001; Qin et al., 2006). In particular, stimulation of ApoE expression enhances the clearance of soluble β-amyloid and reverses multiple deficits in an AD mouse model (Cramer et al., 2012), thus suggesting a role for ApoE in CNS neuroprotection. Extensive evidence for such neuroprotective roles has been accumulated: in *in vitro* studies, ApoE and ApoE mimetic-peptides exerted a neuroprotective effect and ApoE fragments (Tolar et al., 1999) protected neurons from excitotoxic insults, oxidative stress, β-amyloid toxicity, and promoted an anti-apoptotic signaling (Miyata and Smith, 1996; Drouet et al., 2001; Aono et al., 2002; Fuentealba et al., 2009).

In vivo, both ApoE and ApoE-derived peptides have shown neuroprotective properties in models of cerebral hemorrhage, traumatic head injury and AD, effects partly related to ApoE-mediated down-regulation of the inflammatory response caused by microglia activation (Laskowitz et al., 2001; Lynch et al., 2001; Guo et al., 2004; Cramer et al., 2012).

Microglia are an important source of ApoE. This protein is synthesized and secreted by microglial cell lines (Bales et al., 2000; Xu et al., 2000), as well as by primary microglia (Saura et al., 2003; Mori et al., 2004; Qin et al., 2006). In addition, secreted ApoE is one of the molecules involved in neuroprotection against β-amyloid neurotoxicity mediated by microglia conditioned medium (Qin et al., 2006). Although the modulation of ApoE secretion by microglia appears to be crucial for its rapid regulation in the CNS, little

is known about the physiological regulation of microglial-ApoE secretion by neuronal stimuli and how this regulation is modified in inflammatory or neurodegenerative conditions.

1.9 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory loss and dementia. The key pathological hallmarks include deposition of beta amyloid (Aβ) plaques within the brain parenchyma and neurofibrillary tangles in neuronal cell bodies. Analysis of inherited forms of the disease has demonstrated the key role of Aβ homeostasis in AD pathogenesis (Mandrekar-Colucci and Landreth, 2010). Aβ is liberated from the endogenous transmembrane amyloid precursor protein (APP) after the sequential proteolytic processing by two protease complexes β and γ secretases (Zhang et al, 2012 libro). The most common Aβ peptide found in senile plaques is the 42-residue peptide ($A\beta_{42}$), which also has the strongest propensity for spontaneous aggregation in solution forming large highly ordered β-pleated sheet structures known as amyloid fibrils (Burdick et al., 1992), of which both diffuse and dense core amyloid plaques are composed (Mandrekar-Colucci and Landreth, 2010).

In familial forms of AD, increased synthesis and deposition of AβPP-Aβ is due to mutations in the amyloid beta precursor protein (AβPP), presenilin 1 (PS1), and PS2 genes, or inheritance of the Apolipoprotein E ε4 (ApoE-ε4) allele. In sporadic AD, which accounts for 90% or more of the cases, the cause of AβPP-Aβ accumulation is still debated. However, evidence suggests that impairments in insulin/IGF signaling dysregulate AβPP expression and protein processing, leading to AβPP-Aβ accumulation (de la Monte and Tong, 2013).

In addition neurofibrillary tangles, dystrophic neurites, and neuropil threads represent neuronal cytoskeletal lesions that correlate with dementia in AD. These structural lesions contain aggregates of hyperphosphorylated, ubiquitinated, insoluble fibrillar tau. Tau becomes hyperphosphorylated due to inappropriate activation of kinases such as GSK-3β , cyclin-dependent kinase 5 (cdk-5), and c-Abl, or inhibition of protein phosphatases 1 and 2A. Neuronal accumulations of fibrillar tau disrupt neuronal cytoskeletal structure and function, and impair axonal transport and synaptic integrity. Ubiquitination of hyper-phosphorylated tau, together with eventual dysfunction of the ubiquitin-proteasome system, exacerbate the accumulations of insoluble fibrillar tau. Fibrillar tau exerts its

neurotoxic effects by increasing oxidative stress, ROS generation, neuronal apoptosis, mitochondrial dysfunction, and necrosis.

Tau gene expression and phosphorylation are regulated by insulin and IGF, and impairments in insulin/IGF signaling contribute to tau hyper-phosphorylation due to over-activation of specific kinases, e.g. GSK-3β and reductions in tau gene expression. Attendant failure to generate sufficient normal tau protein, vis-a-vis accumulation of hyper-phosphorylated insoluble fibrillar tau likely promotes cytoskeletal collapse, neurite retraction, and synaptic disconnection (de la Monte and Tong, 2013).

Although plaques are universally present in AD, their presence does not directly correlate with clinical dementia; however neurofibrillary tangles demonstrate a better correlation with both neuronal loss and dementia. These observations suggest that tau may be part of a downstream cascade that directly leads to neurodegeneration (Kettenmann and Ransom, 2012).

Despite our advanced knowledge of the molecular basis of Alzheimer's disease this condition remains incurable.

However, it is now well accepted that Alzheimer's disease is marked by neuroinflammatory events involving glia, including astrocytes and, in particular, microglia (Mosher and Wyss-Coray, 2014).

The recent failure of clinical trials based on passive immunotherapy (Bapinezumab and Solanezumab) demonstrate how challenging is to find an efficient cure (Callaway, 2012). The main obstacle to an effective therapy seems to be the time of clinical diagnosis, because at this point the brain has already suffered a prolonged and perhaps irreparable damage. In fact studies on biomarkers in familial and sporadic form of AD demonstrate that the disease starts decades prior to its clinical presentation (Jack et al., 2010; Sperling et al., 2011; Bateman et al., 2012; Do Carmo and Cuello, 2013).

1.10 STUDYING ALZHEIMER'S DISEASE IN TRANSGENIC RATS

Given the progressive and predictable evolution of the pathology and the difficulty in studying AD neuropathology in human "pre-clinical" stages, the use of transgenic models offers the opportunity to identify biomarkers crucial in detecting the disease at the early, "silent" and asymptomatic stage, when therapeutic approaches hopefully have more chance of success.

Etiology, time dependent progression of the pathology as well as structures and cells similar to the human pathology are all multiple aspects that an ideal transgenic model should mimic. Moreover the model should allow suitable learning and memory tests, analysis of body fluids (such as cerebrospinal fluid) and imaging.

Of course a good model should not present visual or motor system impairments (caused by nonspecific transgene or strain effects) that can be falsely confused as AD deficits (Do Carmo and Cuello, 2013).

Despite some technical drawbacks like the more difficult gene injection in pronuclei, the low embryo's survival following injection and the less available tools for manipulating the genome, rats offers multiple advantages compared to mice. The rat is physiologically and genetically closer to humans than mice. Its larger body and brain size make surgical procedures and pharmacological manipulation easier to perform. Another advantage is their richer behavioral display that enables a more accurate assessment of the impact of the pathology on cognitive outcomes and a better assessment of the therapies' potential in longitudinal studies (Do Carmo and Cuello, 2013).

Since the early 2000s a wide number of transgenic rats have been generated based on the expression of human genes relevant for early-onset familial AD, e.g. wild type or mutated APP and mutated PS1. The expression of these transgenes is controlled by different neuronal promoters, thus resulting in different expression strength and patterns. The model could also differ for their inbred or outbred genetic background that could really influence the pathogenesis. Other differences can be due to the method chosen to introduce the transgene in the fertilized eggs (pronuclear injection of DNA or lentiviral delivery) and differences in phenotype can be explained by the presence of single, double or triple transgenes (Do Carmo and Cuello, 2013).

The first transgenic rat models of AD showed intracellular Aβ (iAβ) accumulation but no extracellular plaques. Some of them, however, displayed synaptic dysfunction (LTP and behavior) supporting the view that cognitive deficits are independent of plaque formation but correlate better with Aβ oligomers and other Aβ species. Thus they contributed in

suggesting the role of iAβ in the amyloid cascade at the early, pre-plaque phase of the amyloid pathology (Do Carmo and Cuello, 2013).

1.10.1 Rat models of amyloid pathology presenting mature plaques

The first model characterized by amyloid plaques was achieved in homozygous double transgenic rats Tg478/Tg1116 expressing hAPP695 carrying the Swedish and Swedish/London mutations. These rats show increased APP, Aβ40 and Aβ42 load and by 17–18 months of age diffuse plaques were present (Flood et al., 2009). By introducing a third transgene carrying a human mutated presenilin gene, the resulting PSAPP rats (also named Tg478/Tg1116/Tg11587) showed diffuse plaques starting from the age of 9 months (Liu et al., 2008; Flood et al., 2009). Abundant diffuse plaques are present in the cortex, hippocampus, olfactory bulb, thalamus and hypothalamus but not in the cerebellum or brain-stem. Unfortunately only few compact plaques are detectable in the hippocampus even at 22 months of age and, in addition, no vascular Aβ deposits can be observed. These rats show astrocytic and light microglial activation as well as tau hyperphosphorylation around compact plaques. Moreover they show behavior deficits already detectable at 7 months of age, in absence of plaques, that correlate with Aβ42 load in the hippocampus. The drawbacks are that these rats lack neurofibrillary pathology and neuronal loss. Moreover they show a tendency towards premature death due to kidney disease, hypertension and immunosuppression, that are probably a consequence of genetic disturbance caused by the presence of the triple transgenes (Do Carmo and Cuello, 2013).
The McGill-R-Thy1-APP rat model is the only model able to reproduce extensive AD-like amyloid pathology with a single transgene (Leon et al., 2010). This model expresses the hAPP751, bearing the Swedish and Indiana mutations under the control of the murine Thy1.2 promoter. In the McGill-R-Thy1-APP transgenic rat, a single transgene is able to produce human APP expression specifically in AD-relevant areas of the brain without cerebellar and peripheral tissue expression. The presence of a single transgene with a low copy number makes this rat the least genetically aggressive AD transgenic model developed so far (Do Carmo and Cuello, 2013).

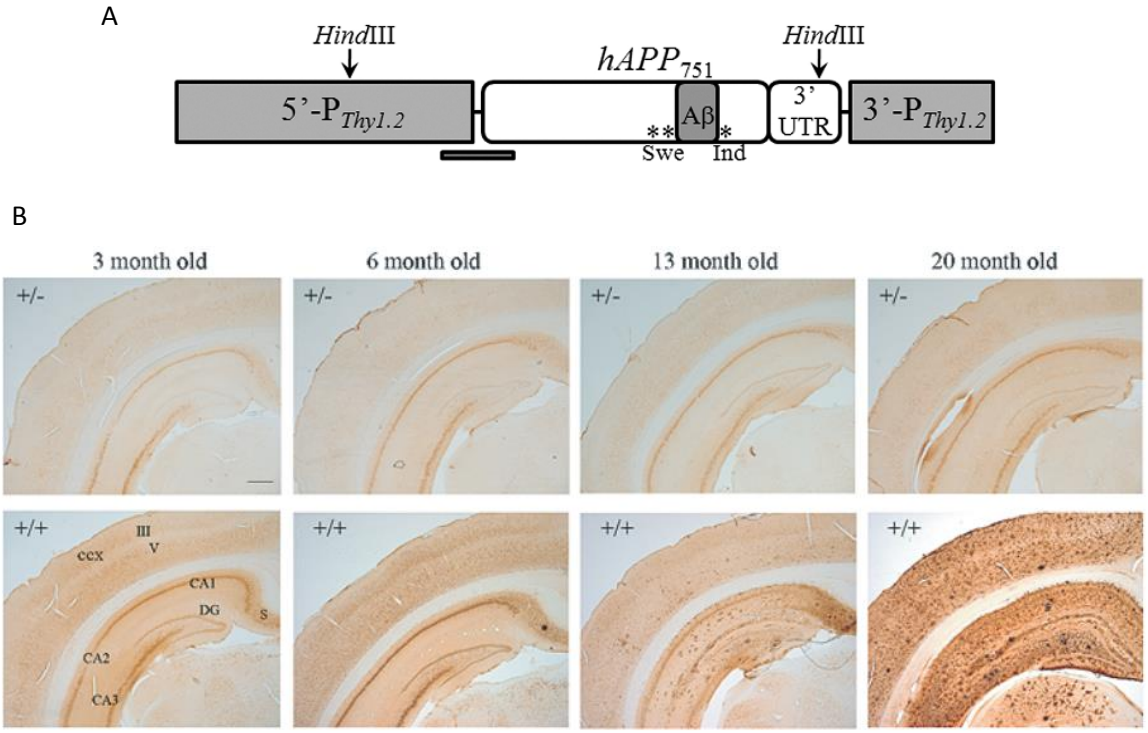

Fig.12. Generation and characterization of the transgenic animals and temporal evolution of the Aβ pathology in McGill-R-Thy1-APP rats. (**A**) Thy1-AβPP construct showing the cloning of hAβPP751 variant (containing the Swedish and Indiana mutations) under the control of the murine Thy1.2 promoter. (**B**) The progression of the amyloid pathology is illustrated in hemi- (upper panels) and homozygous (lower panels) Tg rats (Aβ immunoreactivity dectected with McSA1 antibody). Intraneuronal accumulation of Aβ is well established by 3 months of age, and both lamina layers III and V of the cerebral cortex (ccx) and hippocampus appear intensely stained. The earliest mature amyloid plaques appear in the subiculum (S) of homozygous rats at 6-9 months of age. By 13 months of age, the extracellular amyloid deposition was found extended to most of the areas of the hippocampus and spreading to cortical areas. Modified rom (Leon et al., 2010).

McGill-R-Thy1-APP rats display iAβ as soon as one week post natal in the cortex and hippocampus in both hemi and homozygous animals. The pathology is dose-dependent as, in homozygous animals, iAβ accumulation leads to progressive neuritic plaque deposition starting from 6–9 months old. Hemizygous rats develop no or very few plaques at much later stages. The anatomical spreading of plaques coincides with that observed in human AD, starting from the subiculum and expanding to the entorhinal cortex and hippocampus. The AD-like pathological phenotype also includes the presence of transmitter-specific dystrophic neurites (Leon et al., 2010). It is of significance that the McGill-R-Thy1-APP rat presents progressive behavior impairments in the Morris Water Maze test starting at 3 months of age while no amyloid plaques are yet present. The deficits are transgene-dose-

dependent and they correlate with the abundance of the 12kDa 6E10-immureactive band likely corresponding to a combination of Aβ trimers and the APP C-terminal fragment, C99. This observation reinforces the hypothesis on the impact of oligomeric iAβ in cognition (Shankar et al., 2008). These rats were also used to study metabolite levels by magnetic resonance spectroscopy in the dorsal hippocampus and frontal cortex. The findings demonstrate complex metabolite alterations during the progression of the amyloid pathology, different from those observed during normal aging (Nilsen et al., 2012). MRI imaging on these rats also showed marked brain shrinkage, which is more evident for the hippocampal complex and resting-state connectivity impairment. Consistent with these observations, McGill-R-Thy1-APP rats display impairments in firing rates for place discrimination of spatial context and a very compelling *in vivo* impairment in hippocampal LTP formation at preplaque stages (Do Carmo and Cuello, 2013).

More recently, a bigenic TgF344-AD rat has been created (Cohen et al., 2013). These rats express hAPP695, with the Swedish mutation, and PS1ΔE9 under the control of the strong murine PrP promoter. These rats demonstrate strong age-dependent accumulation of iAβ, soluble and insoluble Aβ40 and Aβ42 peptides and thioflavin positive amyloid plaques. The amyloidosis is associated with hyperactivity in the open-field as well as age dependent deficits in spatial learning and memory as assessed with the novel object recognition and the Barnes maze tasks. Surprisingly, as it was never seen before in other transgenic rat models of AD and even in transgenic AD mouse models expressing APP and PS1 mutations, by 16 months of age these rats present Gallyas-positive structures resembling NFTs seen in human AD. These structures contain p-tau as detected with several p-tau antibodies. The observed amyloidosis and tauopathy are accompanied by neuronal loss. These rats also present glial activation as early as 6 months old, before appreciable extracellular Aβ deposition (Cohen et al., 2013).

Despite their obvious limitations, transgenic rat models will be of great assistance in the search of potential biomarkers signaling an early, preclinical, pathology and in the search and validation of novel therapies. From the experimental point of view, they have already shown to be of significant advantage for *in vivo* electrophysiology, imaging, proteomics, epigenetics and in the future for optogenetic studies (Do Carmo and Cuello, 2013).

1.11 M1/M2 PHENOTYPE

1.11.1 Peripheral macrophages and their different activation states

The initial cell status, as already activated or not, and the type of the activating stimulus (e.g. ageing, injury or chronic disease) play a significant role in determining the spectrum of molecules that are secreted by a macrophage (Gordon, 2003).

Macrophages activation on one hand is potentially helpful to the organisms, for example in killing pathogens by the expression of high levels of pro-inflammatory cytokines and an enhanced microbicide capacity, on the other can also be harmful leading to a detrimental profile associated with tissue damage (known as 'classical activation' or M1). Classical activation has been the activation state most widely explored in animal models.

Furthermore, depending on the type of injury or insult, activated macrophages can express a different profile, also known as 'alternative activation' (M2) (Mosser and Edwards, 2008).

Alternative activation was originally defined following exposure to the Th2 cytokine, interleukin (IL)-4, and upregulation by macrophages of the mannose receptor (Stein et al., 1992). The latter can bind structures on the surface of viruses, bacteria and fungi causing their phagocytosis. Macrophages activated by the Th2 cytokines IL-4 and IL-13 are implicated in different physiological and pathological conditions including homeostasis, inflammation, allergy, malignancy and repair. That's the reason why the M2 category has been further divided into functions relating, first, to tissue repair and wound healing and, second, a state of acquired deactivation (Table 2) (Boche et al., 2013).

1.11.2 What about microglial functional profiles?

1.11.2a Evidences from animal studies

Researchers have worked on differentiating *in vitro* distinct microglial phenotype, functionally classified as cytotoxic (M1), reparative (M2), and even a third, "deactivated" form (Colton, 2009; Michelucci et al., 2009; Moon et al., 2011), thus generating data analogous to those obtained by studying peripheral macrophages *in vitro* (Gordon, 2003). But, in contrast to peripheral macrophages, the mechanisms regulating microglial phenotype in the CNS are nowadays still poorly understood. Microglia show a range of functional phenotypes that broadly correspond to M1/M2 activation of macrophages with M1 microglia often associated with acute infection, and M2 cells playing a role in tissue

remodelling, repair, and healing (Goldmann and Prinz, 2013). This classification is based on the expression of pro- or anti-inflammatory cytokines as well as pro- or anti-inflammatory receptors as a result of microbial products or damaged cells. In particular, interferon gamma (IFN-γ) and lipopolysaccharide (LPS) polarize microglia towards the M1 state and induce the release or expression of interleukin- (IL-) 1, IL-6, IL-12, IL-23, and inducible nitric oxide synthase (iNOS). On the other hand, the presence of IL-4, IL-10, and IL-13 turns microglia into M2 cells, producing IL-10 and expressing arginase 1 (Goldmann and Prinz, 2013).

	M1 (Classic activation)	*M2 (Alternative activation: wound healing)*	*M2 (Alternative activation: regulatory)*
Alternative terms		Tissue repair	Anti-inflammatory
Stimulus	Interferon-γ, TNF-α	IL-4, IL-13, TREM2?	IL-10, glucocorticoids
Source	Natural killer, T helper 1 lymphocytes.	Granulocytes responding to tissue injury, fungi and parasites (chitin), T helper 2 lymphocytes	Macrophage
Macrophage products	Pro-inflammatory cytokines: IL-1β, TNF-α, IL-6, IL-23 Oxygen free radicals	Extracellular matrix components Arginase 1 Chitinase	TGFβ1, IL-10
Cell surface proteins	MHC II?	Mannose receptor (CD206)	
Functions	Kill micro-organisms and other cellular targets. Phagocytosis Present antigen to lymphocytes. May cause collateral damage to host cells.	Tissue repair/wound healing Phagocytosis Increases production/remodelling of extracellular matrix	Inhibits inflammation Phagocytosis

Table 2. Macrophage activation state. An extrapolation may apply to microglia (Boche et al., 2013).

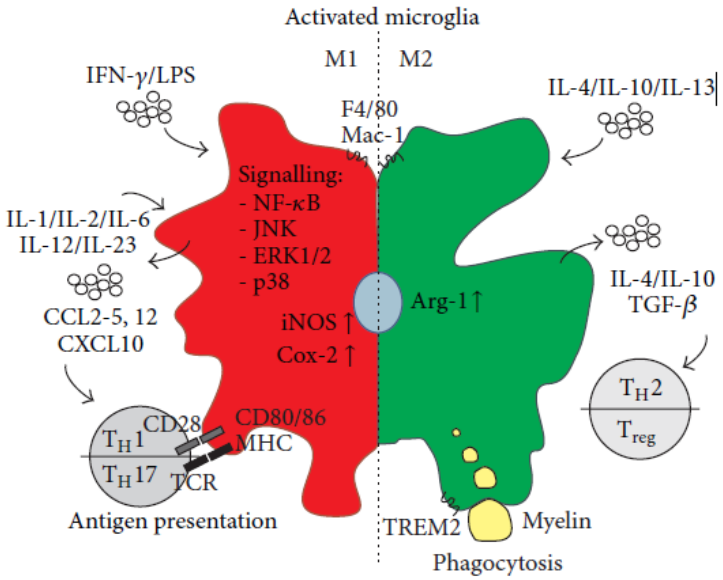

Fig.13. Microglia are activated by diverse stimuli, which define the polarization status of the cell (Goldmann and Prinz, 2013).

Moreover recent studies implicating variants in the TREM2 (Triggering Receptor Expressed on Myeloid cells 2) gene with an increased susceptibility to late onset AD (with an odds ratio similar to that of ApoEε4) are of particular relevance to this discussion (Guerreiro et al., 2013; Jonsson et al., 2013). In particular, TREM2 is considered to be a 'gateway' influencing microglial balance between phagocytic and pro-inflammatory activity. High levels of TREM2 promote alternative activation and phagocytosis, whereas low levels of TREM2 induce a pro-inflammatory state, suggesting that control of microglial activation status is important in AD pathogenesis (Boche et al., 2013). TREM2 is one of the highest expressed receptors in microglia, is >300 fold enriched in microglia vs. astrocytes (Hickman and El Khoury, 2013) and is known to be expressed by microglia surrounding amyloid plaques. It has also been proposed that TREM2 variants cause AD via down regulation of the Aβ phagocytic ability of microglia and by dysregulation of the pro-inflammatory response to these cells. Therefore, identifying TREM2 as a significant risk factor for AD provides insight into the role of microglia in AD (Hickman and El Khoury, 2013).

In general, these observations demonstrate that, in determining the influence of microglia, for example, in the context of neurodegeneration, it is the specific manner in which microglia are activated and the phenotype they adopt that play a crucial role.

However, neurotoxic (M1) and neuroprotective (M2) microglial phenotype cannot be distinguished histopatologically in the brain and the distinction is a simplification and represents the extreme states (Goldmann and Prinz, 2013). As suggested without this capability of discriminate microglial phenotypes, no real progress on functional involvement of putative M1 or M2 microglial subtypes in the cellular pathogenesis of AD can be made (Streit and Xue, 2012).

In the contest of disease, both of these extremes as well as intermediate states may be present. However, the described division in M1 and M2 cells reflects the behavior of microglia concerning their general alternative promotion of tissue injury or repair (Goldmann and Prinz, 2013).

1.11.2b Focus on human brain tissue studies

Considering the context of traumatic brain injury and stroke, M1 polarized microglia has been identified, characterized by an upregulation of IL-1, IL-6 and TNF-a cytokines. On the contrary, the analysis of microglial profile in chronic neurodegenerative diseases appears more challenging. However, studies on animal models of chronic neurodegenerative diseases have highlighted an M2 profile of microglia characterized by expression of transforming growth factor (TGF)-b1 and the lack of the typical pro-inflammatory cytokines IL-1β and TNF-a (Cunningham et al., 2002; Perry et al., 2002). It is suggested that, by adopting the M2 profile, microglia avoid bystander neuronal damage (Boche et al., 2006). Inflammation in human AD brain has been associated with M1 activation in the vicinity of the amyloid deposits (Griffin et al., 1989; McGeer et al., 1989; Griffin et al., 1995) mainly observed by immunoreactivity for IL-1β and complement proteins. However, this contrasts with a study showing a significant increase in AD of the genes associated with M2 activation, *AG1* (arginase) and *CHI3L1/CH3L2* (chitinase 3-like 1/2) with no difference in IL-1β mRNA level (Colton et al., 2006). This is consistent with previous observation of the presence of TGF-β1 in AD (Wang, G. et al., 2003); however, neither study indicates whether the proteins have an active role in disease progression (Boche et al., 2013).

A recent study of brain gene expression in ageing and in AD using microarray technology reported changes in several immune/inflammation-related genes with marked upregulation of genes reflecting activation of microglia and perivascular macrophages, particularly those associated with innate immune responses (Cribbs et al., 2012). The transcriptional changes were more related to cognitively normal ageing than to AD, with upregulation of the complement pathway, TLR signaling, inflammasome activation and immunoglobulin receptors, all of them being able to promote microglial release of pro-inflammatory molecules. For some genes, whose expression profiles was below the microarray sensitivity, the qualitative polymerase chain reaction (qRT-PCR) revealed an increased expression of the proinflammatory cytokines IL-1β, IL-6, TNF-a and also the anti-inflammatory cytokine IL-10. Considering that the changes were more strongly associated with cognitively normal ageing than with the transition to AD, the authors suggested that they may be linked with priming of microglia to the subsequent development of AD.

CHAPTER 2:

AIM OF THE THESIS

Microglial cells are the resident macrophages of the CNS and they are involved in important functions in the developing and adult brain, in both physiological and pathological situations.

For a long time microglia has been considered to be involved mainly in neuro-pathological inflammatory processes but recent studies support their intrinsic neuroprotective role, particularly due to secretion of neuroprotective molecules.

In vitro models, are extremely important for investigating the mechanisms of neuroprotection mediated by microglial cells as well as for identifying new potentially neuroprotective molecules. The experiments described here have been performed using rat primary cultures and in particular cerebellar granule neurons and microglial cells, the latter obtained from both neonatal and adult brain. The use of tools, like toxins, able to induce neuronal apoptosis is a useful model to test the neuroprotective effect of microglial secreted factors. A Parkinson-like neurodegeneration *in vitro* model has been used here by treating cerebellar granule neurons with the toxin 6-hydroxydopamine (6-OHDA).

It is well known from several previous *in vitro* experiments that microglia conditioned medium (MCM) is neuroprotective towards neurons treated with this type of toxin and that at least some of the protective factor(s) are peptidic in nature. However, their specific neuroprotective roles remain relatively unknown in literature.

To begin to fill this gap we decided to analyze the MCM through a proteomic approach that gave the opportunity to identify all the proteins secreted by microglial cells. Among them we focused the studies on Superoxide dismutase 1 (SOD1), an extremely important enzyme for the neuronal redox balance, and Apolipoprotein E, involved in several brain functions, spanning from cholesterol transport to amyloid beta clearance.

The purpose of the thesis has been to dissect the microglial "secretoma" focusing the studies on clarifying the mechanisms of SOD1 and ApoE release and their potential neuroprotective role. Moreover, during my PhD I had the opportunity to spent one year at the McGill University in Montréal, where the project was aimed at the characterization of microglial cells in a rat model of Alzheimer's disease.

So three aims will be discussed:

1) **Microglial SOD1 release and mechanism of neuroprotection**

Pathway involved in SOD1 secretion, time dependence of the release, its modulation with LPS and BzATP, neuroprotective effect on CGNs with or without SOD1 inhibitors. Experiments with exogenous SOD1 added to a non-conditioned medium, SOD1 overexpression/silencing and experiments of calcium imaging.

2) **Microglial ApoE release and its mechanism of neuroprotection also with the use of co-culture system**

Pathway involved in ApoE secretion, time dependence of the release, its modulation with LPS and ATP. Neuronal stimulation of ApoE release (through their conditioned medium and in co-culture conditions). Experiments with exogenous ApoE and with siRNA to test its neuroprotective effect against 6-OHDA toxicity.

3) **Microglial M1-M2 phenotype in McGill-R-Thy1-APP rats**

Using adult microglial cells I analysed, by qRT-PCR, differences in M1/M2 phenotype between microglial cells isolated from control and McGill-R-Thy1-APP rats, the only model able to reproduce extensive Alzheimer's -like amyloid pathology with a single transgene.

These novel information are relevant to stimulate further studies of microglia-mediated neuroprotection in *in vivo* models of neurodegenerative diseases.

CHAPTER 3:

MATERIALS AND METHODS

3.1 Microglial cell cultures and Microglial-Conditioned Medium (MCM) preparation

Microglial secondary cultures were prepared from mixed primary glial cultures from newborn Wistar rat (Harlan Italy, Udine, IT) cerebral cortices, as previously described (Polazzi et al., 2001). All animal experiments were authorized by a local bioethical committee and performed according to Italian and European Community laws on animal use for experimental purposes.

Brain tissue were cleaned from meninges, trypsinized (250 μg/ml, Sigma-Aldrich) for 15 min at 37°C and centrifuged for 3-4 min at 1500 rpm (ALC Multispeed Centrifuge PK121). The pellet was re-suspended in a small volume of DNase I (20 μg/ml, Sigma-Aldrich) and Trypsin Inhibitor (80μg/ml, Sigma-Aldrich) solution for mechanical dissociation with a Pasteur. After an additional centrifugation at 1500 rpm for 10min, cells were seeded on poly-L-lysine (10 μg/ml; Sigma-Aldrich)-coated flasks (75 cm^2). Mixed glial cells were cultured for 7–8 days in Basal Medium Eagle (BME; Life Technologies) supplemented with 10% heat-inactivated Fetal Bovine Serum (hiFBS, Life Technologies), 2 mM glutamine and 100 μM gentamicin sulfate (Sigma-Aldrich).

After 7 days *in vitro* (DIV) microglial cells were harvested from mixed glial cultures by mechanical shaking, re-suspended in serum-free BME and plated on uncoated 35-mm Ø dishes (1.5 x 10^6cells/ 1.5 mL medium/ well). Cells were allowed to adhere for 30 min and then washed to remove non-adhering cells. Microglial cells were stimulated with Brefeldin A (BFA), lipopolysaccharide (LPS) or 2'-3'-O-(4-benzoyl-benzoyl)-ATP (bz-ATP) (all chemicals were from Sigma-Aldrich).

For the evaluation of SOD1 release, microglial cells were plated on uncoated 12-well plates at a density of 7.5 x 10^5 cells/well and stimulated with lipopolysaccharide (LPS), rat recombinant interferon-γ (IFN-γ), or both, as well as with adenosine tryphosphate (ATP) at different concentrations (all chemicals were from Sigma-Aldrich).

After different conditioning times, MCM was collected, filtered through 0.22 μm filters and stored at –20°C until used for neuroprotection analysis on CGNs. For Western blot analysis MCM were desalted and concentrated by using Microcon-YM-3 (Millipore), while microglial cells were collected in 2X Loading Buffer (LB, 50 μl/dish). For immunocytochemistry, microglial cells were fixed for 20 min with 4% paraformaldehyde (PFA) in phosphate buffer (PB) at RT and then washed in phosphate-buffered saline (PBS).

3.2 Cerebellar Granule Neurons (CGNs) and Cerebellar Granule Neuron-conditioned medium (CGNm) preparation

Primary CGNs cultures were prepared from 7-day-old Wistar rats, as previously described (Polazzi et al., 2001). Briefly, cells were dissociated from cerebella and plated on 96-well plates or 35 mm Ø dishes, previously coated with 10 µg/mL poly-L-lysine (respectively 0,12 x 10^6 cells/well and 2,4 x 10^6 cells/well) in BME with 10% hiFBS (Life Technologies), 2 mM glutamine, 100 µM gentamicin sulfate and 25 mM KCl (Sigma-Aldrich). Sixteen hours later, 10 µM cytosine arabino-furanoside (Sigma-Aldrich) was added to avoid glial proliferation. After 7 DIV, differentiated neurons were shifted to serum-free medium with or without 20 µM 6-OHDA (Sigma-Aldrich).

The neuroprotective effect of MCM, exogenous ApoE (human ApoE2, Biovision Inc.) and exogenous SOD1 (Sigma-Aldrich) was tested by co-treating control and 20 µM 6-OHDA-treated CGNs with either microglial conditioned medium or increasing doses of exogenous ApoE/SOD1. Specific SOD inhibitors, ammonium tetrathio-molybdate and disulfiram, or the extracellular calcium-chelating agent ethylenediaminetetraacetic acid (EDTA) were examined to block the neuroprotective effect of exogenous SOD1 as well as that of microglia-secreted SOD1 present in MCM (all chemicals from Sigma-Aldrich). The possible SOD1 neuroprotection was also studied in other classical models of CGNs neurotoxicity, i.e. the shift of differentiated neurons to a low potassium concentration (5 mM KCl) for 24 h or the chronic (24 h) exposure of neurons to 100 µM glutamate (all from Sigma-Aldrich). Neuronal survival was analyzed by using MTT assay or nuclei counting after Hoechst staining. For microglia treatments and western blot analysis, 24 h conditioned media (CGNm) were collected, as previously described for MCM.

3.3 Microglia-CGNs co-cultures

For microglia-CGNs co-cultures, two systems were used:

1) *Microglia exposed to CGNs-conditioned medium (CGNm)*: medium was conditioned for 24 h by differentiated CGNs (2,4 x 10^6 cells/1 mL medium/well in 35-mm Ø dishes at 7 DIV) in serum-free BME 25 mM KCl with or without 20 µM 6-OHDA. All media were used to treat microglial cells (1.5 x 10^6 cells/1 mL medium/well in 35-mm Ø dishes) for 24h in control conditions, as well as with BFA for 4 h, LPS 100 ng/mL for 24 h or Bz-ATP 20 µM for 4 h. Media conditioned by microglia in presence of CGNm in the different experimental conditions were then collected, stored at -20°C, concentrated as previously described, and used for western blot analysis;

2) *CGNs-microglia co-cultures*: microglial cells (1.5 x 10^6) were plated on differentiated CGNs (2,4 x 10^6 cells/well in 35-mm Ø dishes at 7 DIV) in the same medium (1 mL serum-free BME at KCl 25 mM) with or without 20 µM 6-OHDA for 24 h. Conditioned media were then collected and stored at -20°C, until used. For western blot analysis, cells were collected in 2X LB (100 µl/dish), while for immunofluorescence, cells were fixed for 20 min with 4% PFA.

3.4 Microglial transfection

Microglial cells were transfected with the mammalian expression vector pcDNA3 (Invitrogen) containing full-length cDNA encoding wild-type or mutated (G93A) human SOD1 (pcDNA3-SOD1wt/G93A). Alternatively they were transfected with the vector pEGFP-C1 (Clontech) containing full-length cDNA encoding wild type or mutated (G93A/A4V) human SOD1 (pEGFP-SOD1wt/G93A/A4V). All plasmid were a kind gift from Prof. Angelo Poletti (Tortarolo et al., 2004; Sau et al., 2007). The vector pmaxGFP® (Amaxa, Cologne, Germany) was used both as a plasmid control and as a test for transfection efficiency.

Moreover two different commercially available siRNA (Sigma-Aldrich) against SOD1, previously tested for their efficacy by the Rosetta bioinformatics system, were used to silence SOD1 microglial expression. In general, transfection was performed using LipofectAMINE 2000 Reagent (Invitrogen), following the manufacturer's protocol, in OptiMEM/serum-free BME (Invitrogen) without antibiotics for 4 h; the medium with lipid-DNA complex was then replaced with serum-free BME. Twenty-four hours after plasmid transfection, MCM were collected, cleared of contaminating cells debris by filtration (through 0.22-µm filters) or by centrifugation (10,000 *g* for 5 min), and stored at -20°C until use for Western blot and neuroprotection analysis on CGNs.

In order to silence ApoE expression in CGN-microglia co-cultures, microglial cells were transfected with a commercially available siRNA against rat ApoE (previously tested for its efficacy by the Rosetta bioinformatics system) or with a universal siRNA Negative Control (Sigma-Aldrich). Transfection was performed on microglial cells in suspension by using LipofectAMINE 2000 Reagent in OptiMEM/serum-free BME (all from Life Technologies) without antibiotics, following the manufacturer's protocol, and immediately plated on differentiated CGNs in 35-mm Ø dishes. The medium with the lipofectamine-DNA complex was then replaced with serum-free BME. Twenty-four hours following

siRNA transfection, co-cultures were exposed to 20 µM 6-OHDA for additionally 24 hours. For Hoechst staining and immunocytochemistry cells were fixed for 20 min with 4% PFA. For ApoE silencing in rat primary microglia only, cells were transfected as described above; after 4 h, the medium with the lipofectamine-DNA complex was replaced with serum-free BME. Twenty-four or 48 hours following siRNA transfection, MCM were collected, cleared of contaminating cells by filtration (with 0.22 µm filters) or centrifugation (10,000 g for 5 min), and stored at –20°C until used for Western blot analysis.

3.5 Western Blot Analysis

To analyze the level of proteins released into the media or expressed by microglial cell cultures after different times of conditioning and/or after treatments with different stimuli, MCM and cells were collected. Five hundred microliters of MCM for each condition were concentrated and desalted using Amicon YM-3 (Millipore) and resuspended in 10 µl of loading buffer 4X (0.05 M Tris-HCl pH 6.8; 40 g/l sodium dodecyl sulfate; 20 ml/l glicerol; 2 g/l bromophenol blue, and 0.02 M dithiothreitol; all chemicals were from Sigma-Aldrich). In parallel, $1.5/10^6$ microglial cells from each well were collected directly in 50 µl of loading buffer 2X and sonicated using a water bath sonicator. Around 40 µl per lane of concentrated MCM samples or 15 µl of microglial cell samples were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; Bio-Rad Laboratories).

After electrophoresis and transfer to a nitrocellulose membrane (GE Healthcare), the membranes were blocked for 1 h with a blocking solution made of 5% nonfat dried milk (Bio-Rad)/0.1% Tween-20 in phosphate buffer solution (Sigma-Aldrich), pH 7.4, and incubated overnight (O/N) at 4°C with primary antibodies in 0.1% Tween-20/PBS. Then, nitrocellulose membranes were incubated with a secondary antibody for 90 min at 24°C in 0.1% Tween-20/PBS. The labeled bands were visualized using the enhanced chemiluminescence method (ECL; Santa Cruz Biotechnology).

Primary Antibodies used:

Rabbit polyclonal anti-SOD1, ApoE, iNOS (all 1:1,000; Santa Cruz Biotochnology).

Rabbit polyclonal anti-PNP (1:3000, Sigma-Aldrich).

Secondary antibodies used:

Goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2000, Santa Cruz Biotechnology).

3.6 Immunocytochemistry

Control, ATP- treated, BFA-treated microglial cells and co-cultures untreated or treated with 20 µM 6-OHDA for 24 h, were fixed with 4% PFA, washed with PBS and permeabilized with PBS-0.1% Triton X-100. Aspecific sites were blocked with normal goat serum in PBS-0.1% Triton X-100 (all chemicals were from Sigma-Aldrich) for 1h at RT. After several washes, cells were incubated O/N at 4°C with a rabbit anti-ApoE antibody (Santa Cruz), and then with secondary antibodies (anti-rabbit fluorescein isothiocyanate, Sigma-Aldrich) for 1,5 h at RT. For SOD1-LAMP1 staining cells were incubated overnight at 4°C with both rabbit anti-SOD1 and mouse anti-lysosome-associated membrane protein-1 (LAMP-1; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif, USA) and further incubated with the secondary antibodies for 1 h and 30 min at 25 ° C (anti-rabbit fluorescein isothiocyanate for SOD1 and anti-mouse tetramethyl rhodamine isothiocyanate for LAMP-1;Sigma-Aldrich). For Isolectin B_4 microglial staining, co-cultures were pre-incubated with biotin-labeled Isolectin B_4 and then with FITC-labeled extravidin (Sigma-Aldrich). Nuclei were then stained with Hoechst 33258 (0.1 mg/mL; Sigma-Aldrich) for 5 min at RT. Stained cultures were mounted with Vectashield fluorescence mounting media (Vector Laboratories Inc., Burlingame, CA; USA), observed with a fluorescence microscope (Eclipse TE 2000-S microscope; Nikon, Tokyo, JP) and images were acquired with an AxioCam MRm (Zeiss, Oberkochen, Germany) digital camera.

3.7 Viability Assays

The viability of CGNs in culture was evaluated by MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and nuclei counting after Hoechst staining (Polazzi et al., 2001; 2009; 2013). The first assay is based on the reduction of the tetrazolium salt into a colored formazan compound, a reaction that only occurs in viable cells since the chemical reaction is carried out by mitochondrial dehydrogenases. In particular, MTT was added to culture medium at a final concentration of 0.1 mg/mL and following an incubation at 37°C for 20 min in the dark, the formazan precipitate was dissolved in 0.1 M Tris-HCl buffer containing 5% Triton X-100 (all from Sigma-Aldrich) and the absorbance was read at 570 nm in a Multiplate Spectophotometric Reader (Bio-Rad).

For nuclei counting after Hoechst staining, CGNs or CGN-microglia co-cultures were fixed for 20 min with 4% PFA in PB, washed in PBS and incubated for 5 min at RT with 0.1 µg/mL Hoechst 33258 (Sigma-Aldrich). Pictures were acquired with a fluorescence microscope by using a 20X objective and count was performed in 5 randomly selected fields from each 35mm Ø dish. Neuronal survival was evaluated by counting normal, uncondensed nuclei.

3.8 Calcium Microfluorometry

Variations in intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) were monitored through ratiometric microfluorometry using the fluorescent Ca^{2+} detector fura-2 AM (Molecular Probes; Invitrogen, Milano, Italy). For microfluorometric experiments the control bath saline was 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM TES, and 5 mM glucose, pH 7.4, with NaOH and osmolarity adjusted to ~320 mOsm with mannitol. The Ca^{2+}-free extracellular saline was prepared by removing $CaCl_2$ salt and adding 0.5 mM EGTA. When using high (50 mM) K^+ external solutions, salts were replaced equimolarly. Stock solution of SOD1 was diluted in control and Ca^{2+}-free media at the final concentrations of 0.75, 1.5, 3.125, 6.25, and 12.5 U/ml. The 48-hour MCM solution was obtained by dissolving the lyophilized powder in 10 ml of the control bath saline with and without calcium. Before measurements, low-density granular cells seeded in coverslips were loaded with 10 µM fura-2 AM dissolved in standard bath solution for 45 min at 25°C. For microfluorometric analysis cell coverslips were mounted on a perfusion chamber containing 100 µl bath saline. Cells were continuously perfused at a rate of 0.5 ml/min with different salines at room temperature (22–24 °C) as previously described (Benfenati et al., 2011). Measurements of $[Ca^{2+}]i$ were performed using an inverted fluorescence microscope (Nikon Eclipse TE2000U; Nikon, Italy) equipped with a long-distance dry objective (40X) and appropriate filters. The emission fluorescence of selected astrocytes was passed through a 510-nm narrow-band filter and acquired with a digital charge-coupled device camera (VTi; Visi-Tech International Ltd., Sunderland, UK). Monochromator settings, chopper frequency, and complete data acquisition were controlled by QuantiCell 2000 (VisiTech). The excitation wavelength was alternated between 340 and 380 nm with a sampling rate of 0.25 or 0.5 Hz. The fluorescence ratio measured at 340 and 380 nm (F340/F380) was used as an indicator of $[Ca^{2+}]i$ changes. The calibration of the 340/380 ratio in terms of the free Ca^{2+} concentration was based on the procedure previously described (Grynkiewicz et al., 1985).

3.9 SOD1 Activity Determination

SOD1 activity in 20 µl of 48-hour MCM concentrated 2- to 4-fold was determined using the SOD determination kit from Fluka (Sigma-Aldrich). This method is based on the ability of SOD1 to catalyze the dismutation of the anion superoxide, previously produced by xanthine oxidase starting from xanthine and O_2, into molecular oxygen and hydrogen peroxide, which in turn can reduce a highly soluble tetrazolium salt (WST-1) to a formazan dye that can be easily revealed through a spectrophotometer. Since the absorbance of formazan dye is proportional to the amount of anion superoxide, SOD activity is quantified as an inhibition activity by measuring the decrease in color development. For quantification, a standard inhibition curve was prepared with a known concentration of exogenous SOD1 (Sigma-Aldrich) from 1.125 to 10 U/ml and the reaction was followed for at least 20 min.

3.10 Adult microglia cell culture

3.10.1 Animals

The animals were housed in groups of up to three in individually ventilated cages under standard conditions (22°C, 12 h light-dark cycle) receiving food and water ad libitum. All procedures were approved by the Animal Care Committee of McGill University and followed the guidelines of the Canadian Council on Animal Care. At 5 or alternatively 16-17 months of age rats were sacrificed by transcardial perfusion and the brains have been processed for qRT-PCR.

Pre-plaques stage:	**Post-plaques stage:**
5 months	16-17 months
8 rats	8 rats (+/+)
4 WT and 4 TG (+/+)	4 WT and 4 TG (+/+)
All males	TG: ½ females

3.10.2 Preparation

Briefly modified from (Slepko and Levi, 1996)

The animals were deeply anaesthetized with Equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3 ml, i.p., per 100 g body weight) and perfused through the left ventricle with cold filtered PBS using 2 sterile syringes (60ml) in order to minimize the potential contaminations. Each brain was immediately transferred in a falcon tube with ice-cold phosphate buffered saline (PBS with $NaHCO_3$ 0.75g/l , Hepes buffer 10mM, pH 7.4). Under the hood, cortex, hippocampus and cerebellum were dissected and cleared from meninges. Importantly, each cerebral area of every rat was kept and treated separately. After that, the tissues were transferred in a trypsin solution (RPMI-1640 containing 0.25% trypsin, 0.02% EDTA, and 0.01% collagenase type II), minced with a scalpel and incubated at 37°C for 1 h with shaking. Then RPMI-1640 (GIBCO) + 10% FBS (GIBCO) was added and the suspension was centrifuged at 2,000 rpm (Beckman Coulter Avanti® J-E,) at 4°C for 5 min. The pellet was resuspended in RPMI-1640 containing 40 µg/ml DNAse type I (Sigma-Aldrich) and incubated at 37°C for 15 min with shaking. Then ice cold RPMI-1640 was added and the suspension was centrifuged at 2,000 rpm at 4°C for 5 min.

The pellet was resuspended in 1 ml of cold RPMI using a P1000 Gilson pipette, until the suspension was homogeneous. Other 20.4 ml of cold RPMI (reaching 21.4ml in total) and 8.6 ml of isotonic percoll in Hanks' buffer (GE Healthcare and Sigma-Aldrich respectively) were added and the suspension was centrifuged again at 2,000 rpm at 4°C for 20 min.

The pellet was resuspended in cold Hank's buffer without Ca^{2+} and Mg^{2+} (GIBCO) with 2% FBS and centrifuged at 2,000 rpm at 4°C for 5 min. One ml of cold RPMI-1640 has been used for resuspending the last pellet and each suspension was divided in 3 eppendorfs and centrifuge at 2500 rpm (Eppendorf 5418) for 5 min at RT. The supernatants were discarded and the pellets stored at -80° until used.

3.11 RNA extraction and quantitative Real-Time PCR (qRT-PCR)

For total RNA extraction, 300µl of each cell suspension were centrifuged and the pellets were directly lysed using RNeasy Mini Kit (QIAGEN, USA), according to the manufacturer's instruction. RNA were collected in 30 µl of RNase free water and stored at -80°C until used. Total RNA was retro-transcribed with the Omniscript RT Kit (QIAGEN, USA) using an oligo-dT primer to generate cDNA, stored at −20°C until used. Quantification of transcript expression was assessed by qRT-PCR with EvaGreen® (MBI EVOlution EvaGreen qPCR Mix, Montreal Biotech Inc.) using the Eco™ Real-Time PCR system (Illumina Inc., USA) thermal cycler. qRT-PCR measurements were carried out in duplicate with four biological replicates (4 rats/age). Expression of each gene was normalized to the housekeeping gene β-actin as the internal control. qRT-PCR primers were designed using the software Primer Blast.

3.11.1 Primers

PCR products have similar length (80bp ≤ product length ≤ 120bp):

Gene	Accession number	primer	Sequence 5'---3'
Il-6	NM_012589.2	Forward	CATTCTGTCTCGAGCCCACC
		Reverse	GCTGGAAGTCTCTTGCGGAG
Il-1β	NM_031512.2	Forward	CAGCTTTCGACAGTGAGGAGA
		Reverse	TCTGGACAGCCCAAGTCAAG
iNOS	NM_012611.3	Forward	CACAGTGTCGCTGGTTTGAA
		Reverse	CCGTGGGGCTTGTAGTTGAC
COX2	NM_017232.3	Forward	TGGGCCATGGAGTGGACTTA
		Reverse	AGGATACACCTCTCCACCGA
Il-2	NM_053836.1	Forward	CCAAGCAGGCCACAGAATTG
		Reverse	TCCAGCGTCTTCCAAGTGAA
Il-12a	NM_053390.1	Forward	GTGTCAATCACGCTACCTCCT
		Reverse	CTTGGCAGGTCCAGAGACTG
TNFα	NM_012675.3	Forward	ATGGGCTCCCTCTCATCAGT
		Reverse	GCTTGGTGGTTTGCTACGAC
IL-23α	NM_130410.2	Forward	AGGACAACAGCCAGTTCTGTTT
		Reverse	AGAAGGCTCCCCTGTGAAGA

Il-10	NM_012854.2	Forward	TGCGACGCTGTCATCGATTT
		Reverse	TGGCCTTGTAGACACCTTTGT
Il-4	NM_201270.1	Forward	CGGATGTAACGACAGCCCTC
		Reverse	TGGTGTTCCTTGTTGCCGTA
YM1	NM_001191712.1	Forward	ACTTGGACTGGCAATACCCTG
		Reverse	TTTACGCATTTCCTGCACCAG
Arg1	NM_017134.3	Forward	ACAAGACAGGGCTACTTTCAGG
		Reverse	ACAAGACAAGGTCAACGCCA
Mrc1	NM_001106123.2	Forward	TCAACTCTTGGACTCACGGC
		Reverse	GAACGGAGATGGCGCTTAGA
TGFb1	NM_021578.2	Forward	CTGCTGACCCCCACTGATAC
		Reverse	AGCCCTGTATTCCGTCTCCT
GFAP	NM_017009.2	Forward	CAGCTTACTACCAACAGTGCC
		Reverse	GGTTTCATCTTGGAGCTTCTGC
NeuN	NM_001134498.2	Forward	CAGCAGCCCAAACGACTACA
		Reverse	TTCCCGAATTGCCCGAACAT
iba1	NM_017196.3	Forward	CCTCATCGTCATCTCCCCAC
		Reverse	AAGCTTTTCCTCCCTGCAAATC
β-Actin	NM_031144.3	Forward	AGGCATCCTGACCCTGAAG
		Reverse	GCTCATTGTAGAAAGTGTGG
MIP-1a	NM_013025.2	Forward	CGGGTGTCATTTTCCTGACCA
		Reverse	CCTTGCTGCCTCTAATCTCAGG
TREM2	NM_001106884.1	Forward	GGTCACAGAGCTGTCCCAAG
		Reverse	CAGTGCCTCAAGGCGTCATA
ApoE	NM_001270681.1	Forward	GGTCCCATTGCTGACAGGAT
		Reverse	GCAGGTAATCCCAGAAGCGG
LRP1	NM_001130490.1	Forward	AGCTGCTGCGAGTAGACATC
		Reverse	CAGCCGCCATTGTTGATACG

3.12 Statistical analysis

All quantitative data are presented as means ± SE from independent experiments. Statistical significance between different treatments was calculated by using one way analysis of variance (ANOVA) followed by post-hoc comparison through Bonferroni's test or Student's t test. Two-group comparisons were analysed with a 2-tailed Student's t-test. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 4:

RESULTS

4.1

Evidences on microglial SOD1 release and neuroprotection

4.1.1 SOD1 IS CONSTITUTIVELY RELEASED BY MICROGLIAL CELLS

It is well known from previous studies that the exposure of differentiated rat CGNs to the dopaminergic toxin 6-OHDA (20 µM) for 24 h resulted in approximately 50% cell death (Monti et al., 2007; Polazzi et al., 2009).

Interestingly, a medium conditioned by microglia for 2 h (2-hour MCM) did not protect neurons from 6-OHDA toxicity, whereas a media conditioned for 24 and 48 h (24-hour and 48-hour MCM) had a significant neuroprotective action (fig.1A). This suggests that neuroprotective substances released by microglia progressively accumulated in the medium during conditioning. Western blot analysis confirmed that SOD1 was actually present in 24 and 48 h MCM, while it was much less evident after 2 h of conditioning (fig.1B), thus suggesting that SOD1 could play an important role in MCM-mediated neuroprotection.

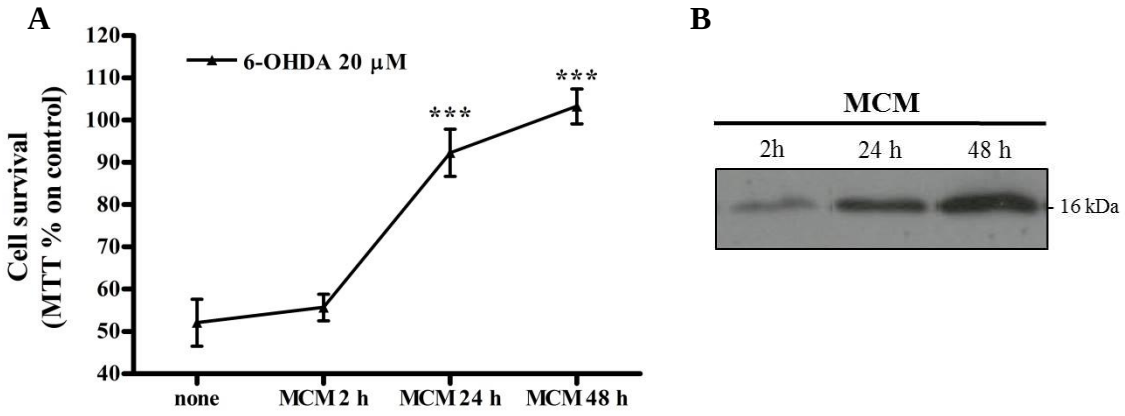

Fig. 1. (**A**) **Neuroprotective effect of MCM**. Cells (CGNs) were treated for 24 h with 20 µM 6-OHDA in the presence or absence of medium conditioned for 2, 24, and 48 h by unstimulated microglial cell cultures (100% of the culture media). Cell viability was determined through MTT assay. Each point is the mean ± SE of at least four different experiments run in triplicate. *** $p < 0.001$ compared to the conditions of treatment with 6-OHDA in non-conditioned medium. (**B**) **Identification of SOD1 release and accumulation in the MCM**. Representative Western blot analysis of SOD1 protein expression in different media conditioned by microglia for 2, 24, and 48 h. (from Polazzi, Mengoni et al., 2013).

In order to estimate the amount of SOD1 in the neuroprotective medium (48-hour MCM), we measured its catalytic activity using a commercially available SOD-assay kit. In parallel, we compared the intensity of Western blot bands derived from a known volume of the 48-hour MCM (i.e. 500 µl) with the intensity of bands obtained by loading known amounts of recombinant SOD1. Both methods yielded similar values, 0.04/0.05 U/ml,

which corresponds to about 10 ng/ml in the 48-hour MCM (according to the activity/concentration relationship given by the producer of the recombinant SOD1).

	SOD1 Activity (U/ml)
SOD1 Determination Kit	**0.057 ± 0.0024**
Western Blot analysis	**0.044 ± 0.0006**

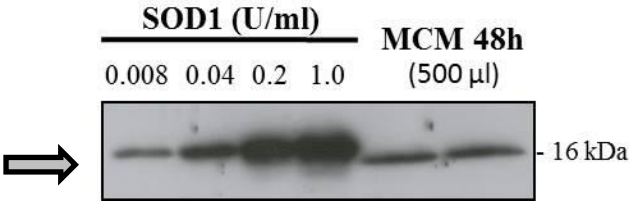

Fig. 2. Determination of SOD1 units in 48-hour MCM through two different methods: analysis of SOD1 protein expression (Western blot) in 48-hour MCM, compared to different known units of SOD1 from human erythrocytes, and determination of SOD activity using a specific kit (from Polazzi, Mengoni et al., 2013).

4.1.2 MODULATION OF SOD1 SECRETION BY MICROGLIAL ACTIVATORS

Considering that the release of SOD1 in the conditioned medium appeared to be constitutive, i.e. related to the normal partially activated *in vitro* microglial conditions, we tested whether a more activated state could modulate SOD1 microglial secretion. Activation of microglia with LPS, IFN-γ, or both did not increase the SOD1 level in a medium conditioned for 24 h and it did not change the intracellular level of the enzyme. On the contrary it induced, as expected, a strong increase in iNOS levels, thus confirming the actual activated state of microglia under these conditions.

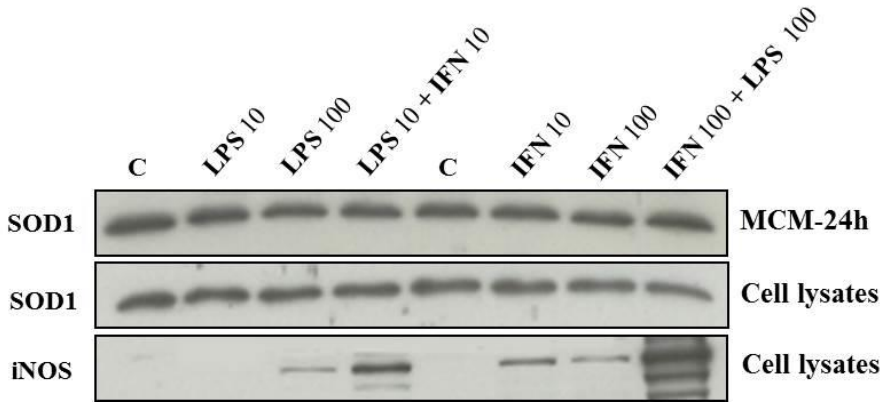

Fig. 3. Constitutive release of SOD1 by microglial cells is not modulated by pro-inflammatory agents. SOD1 protein expression was analyzed by Western blot in different conditioned media and in cell lysates of microglia cell cultures treated or co-treated for 24 h with different concentrations of LPS and IFN-γ. The increase in the intracellular expression of iNOS was also analyzed to confirm microglial activation (from Polazzi, Mengoni et al., 2013).

SOD1 secretion should be atypical since this protein lacks the secretion signal sequence at its amino terminal polypeptide chain. However, its extracellular release has been well documented in several cell types through a mechanism involving a vesicle-related and, in some cases, ATP-dependent pathway (Mondola et al., 2003; Turner et al., 2005; Santillo et al., 2007). Since ATP has been shown to stimulate the extracellular release of the lysosomal compartment content from microglia (Bianco et al., 2005; Takenouchi et al., 2009), we investigated the effect of this purine on microglial SOD1 secretion. As shown in fig.4A, 1 h stimulation with 500µM ATP increased the level of SOD1 in culture medium, thus suggesting that SOD1 could be released by microglial cells through a lysosomal secretory pathway.

To further support this hypothesis, we studied SOD1 co-localization with LAMP-1, a vesicular marker of the lysosomal secretory pathway, using an immunocytochemical approach (Persson et al., 2002). Therefore, we performed double immunofluorescent staining with an antibody against SOD1 and one against LAMP-1. This analysis demonstrated that the finely granular and partially overlapping cytoplasmic distribution of the two proteins in unstimulated microglia shifted towards a clear co-localization in secretory granules after 1 h of ATP stimulation.

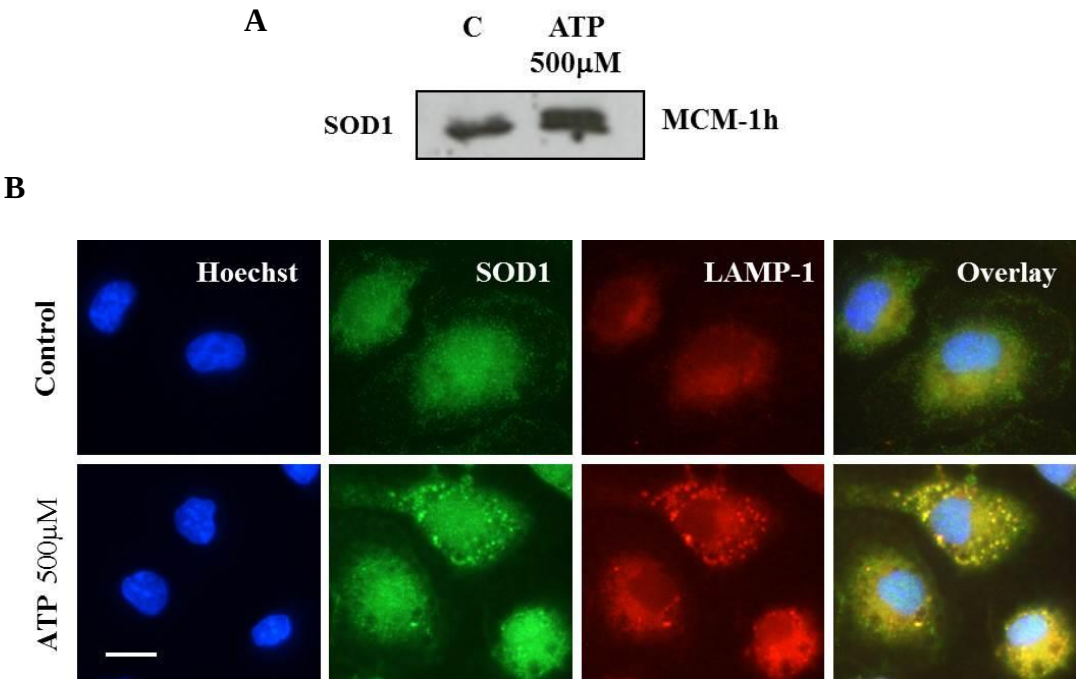

Fig. 4. Constitutive release of SOD1 by microglial cells is increased by ATP (**A**) The microglial release of SOD1 was detected by Western blot in the medium of microglial cell cultures stimulated for 1 h with 500µM ATP. (**B**) Immunocytochemical analysis of SOD1 and LAMP-1 in microglial cell cultures in basal conditions or after stimulation with 500 µM ATP for 1 h. Scale bar = 10 nm . C = Control. (from Polazzi, Mengoni et al., 2013).

4.1.3 SOD1-MEDIATED NEUROPROTECTION

In order to demonstrate a relationship between SOD1 accumulated in the medium and MCM neuroprotection, we added increasing amounts of exogenous SOD1 to a non-conditioned medium and tested it for neuroprotection on 6-OHDA-challenged CGNs. Exogenously added SOD1 was significantly neuroprotective under these conditions, even though at concentrations ~40 times higher than those previously estimated in MCM.

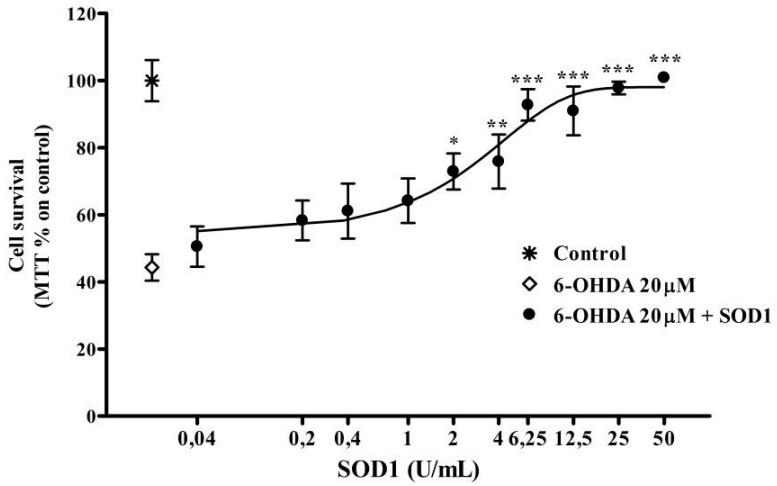

Fig.5. Effect of exogenous SOD1 against 6-OHDA-induced neurotoxicity in CGNs. MTT assay of CGN cultures treated for 24 h with 20µM 6-OHDA in the presence or absence of increasing amounts of exogenous SOD1 (0.04–50 U/ml). Each point is the mean ± SE of eight different experiments run in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to 6-OHDA treatment (from Polazzi, Mengoni et al., 2013).

Contradictory previously published results (Heikkila and Cabbat, 1978; Tiffany-Castiglioni et al., 1982) suggested that SOD1 could inhibit the spontaneous process of 6-OHDA auto-oxidation that leads to generation of superoxide radicals and quinones putatively responsible for neurotoxicity. We thus performed a specific control experiment aimed to demonstrate that, in our conditions, SOD1 neuroprotection was a true biological effect and not simply the result of a purely chemical effect, i.e. of a toxin concentration decrease in the medium.

For this purpose a non-conditioned medium containing 20 µM 6-OHDA was pre-incubated for 2 or 6 h with a strongly neuroprotective concentration of SOD1 (12.5 U/ml), and then this medium was passed through a filter with a molecular cutting of 10 kDa in order to trap SOD1, but not 6-OHDA, thus leaving the toxin in the medium. The resulting medium maintained the same toxic effect of a medium in which 20 µM 6-OHDA has been freshly added. These data showed that pre-incubation with SOD1 did not result in a limited

neurotoxicity of 6-OHDA attributable to a SOD1-dependent chemical modification. In this way we could demonstrate that SOD1-mediated neuroprotection was not due to a non-specific decrease in 6-OHDA neurotoxicity but was the result of a clear neuroprotective effect of this protein.

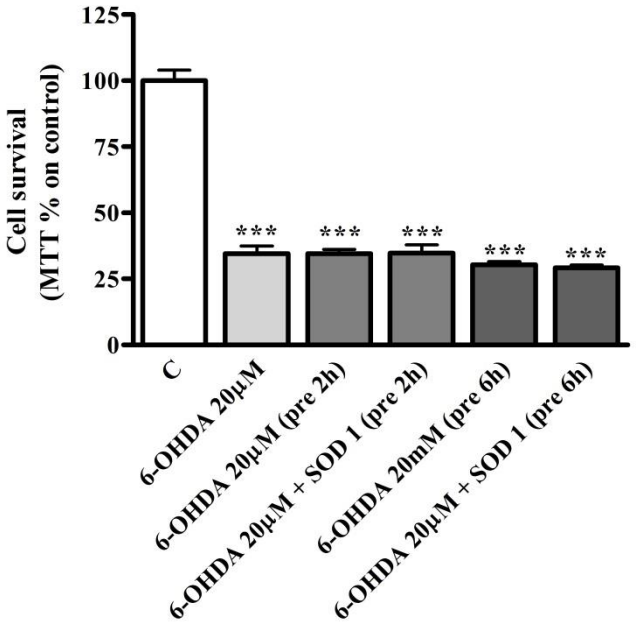

Fig.6. SOD1 neuroprotection is not due to 6-OHDA decreased neurotoxicity. MTT assay of CGN cultures treated for 24h with 20µM 6-OHDA freshly prepared or 20µM 6-OHDA pre-incubated for 2h or 6h at 37 °C with or without SOD1 (12.5 U/ml). Bars are the means ± SE of four different experiments run in triplicate. *** $p < 0.001$ compared to control conditions. Bonferroni's test after ANOVA. (from Polazzi, Mengoni et al., 2013).

4.1.4 SOD1 OVER-EXPRESSION

We decided to transfect our microglial cells with plasmids expressing either human wild-type or mutated (G93A) SOD1. Both wild type and mutated SOD1 were released by microglia (fig.7) but this increased release of SOD1 in the medium conditioned for 24h was irrelevant to neuroprotection. Actually these media did not show any improvement in neuroprotection compared to the 24-hour standard conditioned medium (fig.8), thus confirming that the amount of protein normally released by microglia is enough to grant protection.

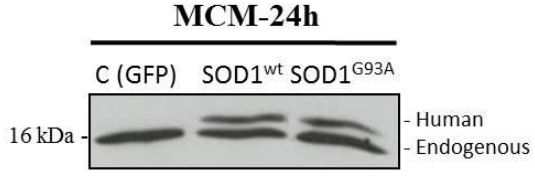

Fig.7. Western blot analysis of SOD1 in 24h-MCM following transfection with plasmids over-expressing GFP or wild-type or mutated (G93A) human SOD (modified from Polazzi, Mengoni et al., 2013).

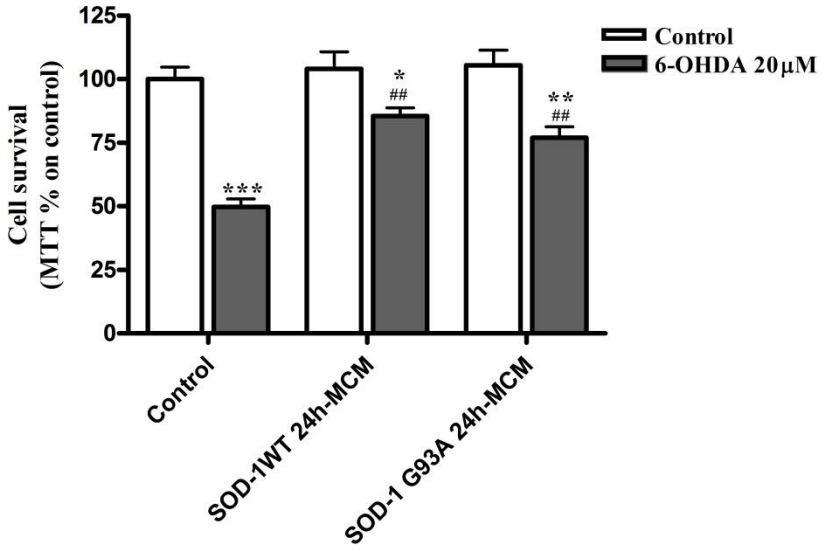

Fig.8. SOD1 over-expression is not improving MCM-24h neuroprotection. MTT assay of CGNs cultures treated for 24h with 20µM 6-OHDA in the presence or absence of a medium conditioned for 24h by control microglial cells or by microglia overexpressing either wild-type or mutated (G93A) human SOD1. Each point is the mean ± SE of 4 different experiments run in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to its own control condition; $^{\#\#}p < 0.01$ compared to control 6-OHDA. Student's t-test. (from Polazzi, Mengoni et al., 2013).

4.1.5 DIFFERENCES IN SOD1 SECRETION BY MICROGLIAL CELLS WHEN EXPRESSING HUMAN SOD1 IN ITS WILD TYPE OR MUTATED FORMS

In a preliminary study we decided to investigate in more detail the differences in secretion between the wild type or mutated (G93A and A4V) forms of SOD1. Mutated forms of SOD1 are less secreted by transfected microglial cells with respect to cells expressing the wild type form. Further studies are required in order to validate this data.

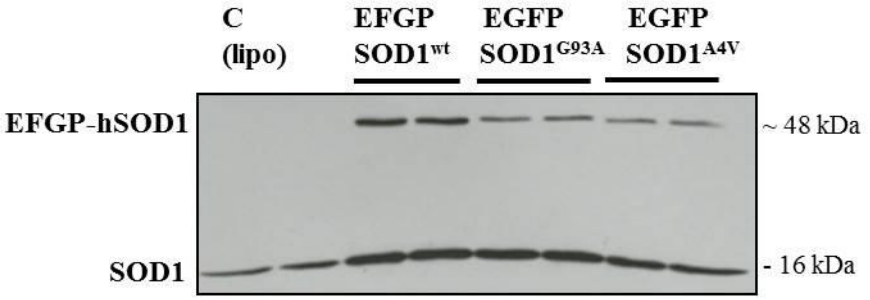

Fig.9. Mutated forms of SOD1 are less secreted by microglia. Western blot analysis showing endogenous SOD1 and transfected EGFP-human SOD1 in 24h-MCM following transfection with pEGFP-c1 plasmid carrying the wild-type or mutated (G93A and A4V) forms of human SOD1. Control cells are treated with Lipofectamine 2000 only.

4.1.6 siRNA-MEDIATED SOD1 SILENCING

In an attempt to demonstrate the specificity of the SOD1 neuroprotective effect in the MCM, we tried to silence SOD1 expression in microglial cells using a siRNA approach. By Western blot analysis we only observed a partial decrease, and not a complete abrogation, of SOD1 in the medium conditioned for 48 h by the siRNA-transfected microglia. Moreover, the MCM maintained its neuroprotective activity suggesting that the siRNA-mediated decrease in SOD1 release did not affect MCM neuroprotection

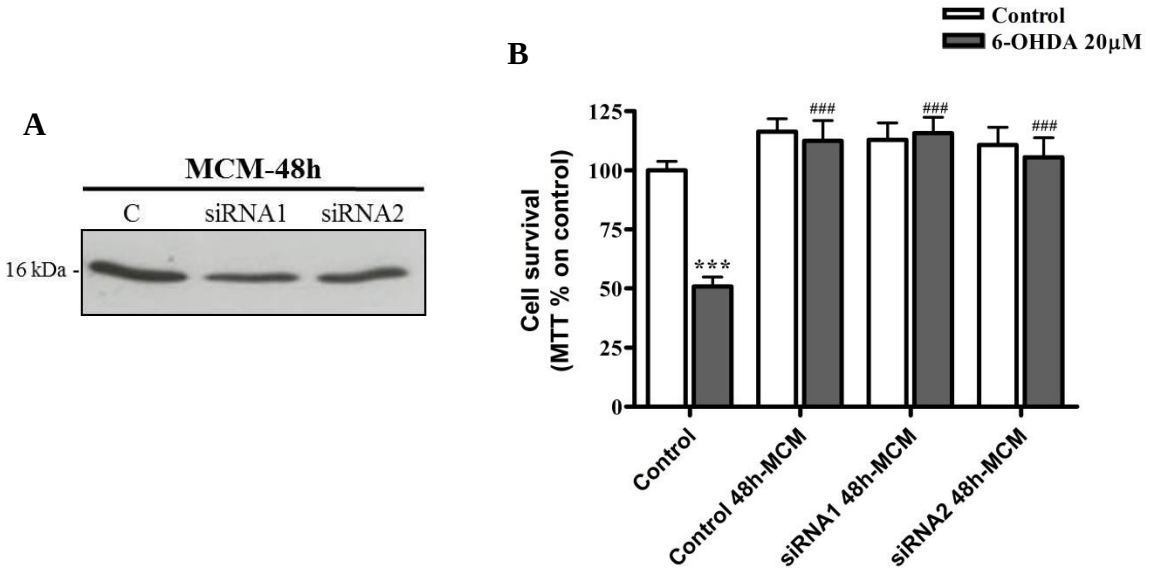

Fig.10. siRNA-mediated SOD1 silencing is not able to counteract MCM neuroprotection. (**A**) Western blot analysis of SOD1 shows a decrease in SOD1 release in 48h-MCM following transfection with two different commercially available SOD1-specific siRNAs. (**B**) MTT assay of CGNs cultures treated for 24h with 20μM 6-OHDA in the presence or absence of a medium conditioned for 48h by control microglial cells or by microglia transfected with each siRNA. Each point is the mean ± SE of 2 different experiments run in triplicate. $^{***}p<0.001$ compared to control; $^{###}p<0.001$ compared to 6-OHDA. Student's t-test (from Polazzi, Mengoni et al., 2013).

4.1.7 PHARMACOLOGICAL INHIBITION OF SOD1 CATALYTIC ACTIVITY

Considering that silencing SOD1 expression was not the appropriate method to successfully block SOD1 in our experimental model, we decided to adopt a pharmacologic approach. In this way we could interfere with the catalytic activity of SOD1, using two SOD1 inhibitors, ammonium tetrathio-molybdate and disulfiram (Forman et al., 1980; Ogra et al., 1999; Juarez et al., 2006), and we showed that both of them were able to fully reverse the effect of neuroprotective concentrations of exogenous SOD1 (fig. 11A, B).

Moreover, these SOD1 inhibitors substantially decreased the neuroprotective effect of 48-hour MCM against 6-OHDA neurotoxicity (fig. 11C,D), thus confirming the neuroprotective role of SOD1 in the MCM.

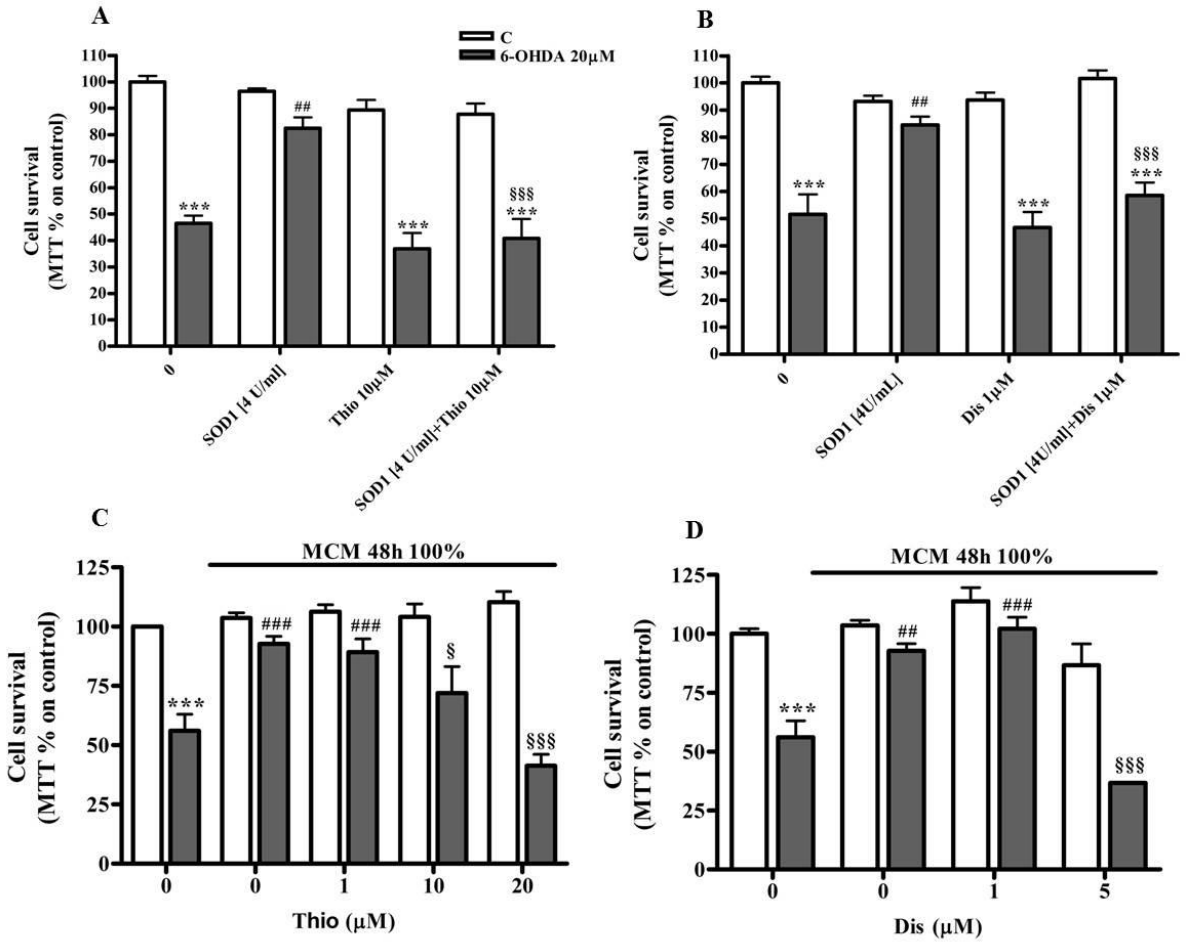

Fig. 11. Two inhibitors of superoxide dismutase, ammonium tetrathio-molybdate (Thio) and disulfiram (Dis), block the neuroprotective effect of exogenous SOD1 and MCM. MTT assay. Ammonium tetrathio-molybdate (Thio) 10µM (**A**) or disulfiram (Dis) 1µM (**B**) was added to CGN cultures treated for 24 h with 20 µM 6-OHDA in the presence or absence of exogenous SOD1 (4 U/ml). Bars are the means ± SE of four different experiments run in triplicate. *** $p < 0.001$ compared to its own control condition; ## $p < 0.01$ compared to the 6-OHDA condition (with neither SOD1 nor the SOD inhibitor); §§§ $p < 0.001$ compared to the parallel SOD1 condition (with 6-OHDA and SOD1 at the same concentration but without the SOD inhibitor).
Thio (1, 10 or 20µM) (**C**) or Dis (1 or 5µM) (**D**) was added to the cultures of CGNs treated for 24h with 20µM 6-OHDA in presence or absence of medium conditioned for 48h by unstimulated microglial cell cultures (100% of the culture media). Bars are the means ± SE of four different experiments run in triplicate. *** $p < 0.001$ compared to control conditions; ## $p < 0.001$, ### $p < 0.001$ compared to 6-OHDA conditions (with neither 48-hour MCM nor the SOD inhibitor); § $p < 0.05$, §§§ $p < 0.001$ compared to the condition with 6-OHDA and 48-hour MCM (without the SOD inhibitor). Bonferroni's test after ANOVA. (from Polazzi, Mengoni et al., 2013).

4.1.8 SOD1 ACTING AS A SIGNALING MOLECULE

In view of previous evidence that released SOD1 could regulate cellular calcium levels in a paracrine way through receptor-mediated interactions in neuroblastoma cells (Mondola et al., 1994; Mondola et al., 2004; Secondo et al., 2008), we analyzed the involvement of a similar mechanism in SOD1-mediated and, more generally, in MCM-mediated neuroprotective action. To this aim, a microfluorometric analysis of intracellular calcium dynamics was performed in 2 mM $[Ca^{2+}]_o$ by measuring changes in the fluorescence emission ratio of fura-2-loaded CGNs. Extracellular challenge with SOD1 (6.25 U/ml) promoted a significant rise in $[Ca^{2+}]_i$. Notably, 90% of all of the granular cells tested responded to SOD1, and the viability of responding and non-responding cells was checked in every experiment by the application of high K^+ (50 mM) external solution (not shown). The $[Ca^{2+}]_i$ responses were variable both in amplitudes and dynamics depending on the presence of the extracellular calcium. The most representative behavior in calcium dynamics in response to SOD1 was a significant increase in $[Ca^{2+}]_i$ signals occurring with a time lag of ~2 min (fig. 12A). The $[Ca^{2+}]_i$ responses were always dependent on $[Ca^{2+}]_o$ presence because omission of extracellular Ca^{2+} caused a rapid reduction of $[Ca^{2+}]_i$ signals as a result of administration of SOD1 (fig. 12A). This result was corroborated by the fact that when perfusion of neurons started in SOD1-containing, Ca^{2+}-free medium there was no response until Ca^{2+} was added to the medium (fig. 12B).

Responses similar to those described for the addition of exogenous SOD1 were elicited by SOD1-containing medium conditioned for 48 h by microglia (fig. 12C, D). Conversion of fluorimetric data into direct evaluation of intracellular calcium levels and statistical analyses confirmed that SOD1-induced calcium signals were significantly higher than at baseline and dependent upon $[Ca^{2+}]_o$ (fig. 12E). Altogether, these data suggest that perfusion with SOD1 promotes complex $[Ca^{2+}]_i$ signals that are dependent on the presence of $[Ca^{2+}]_o$.

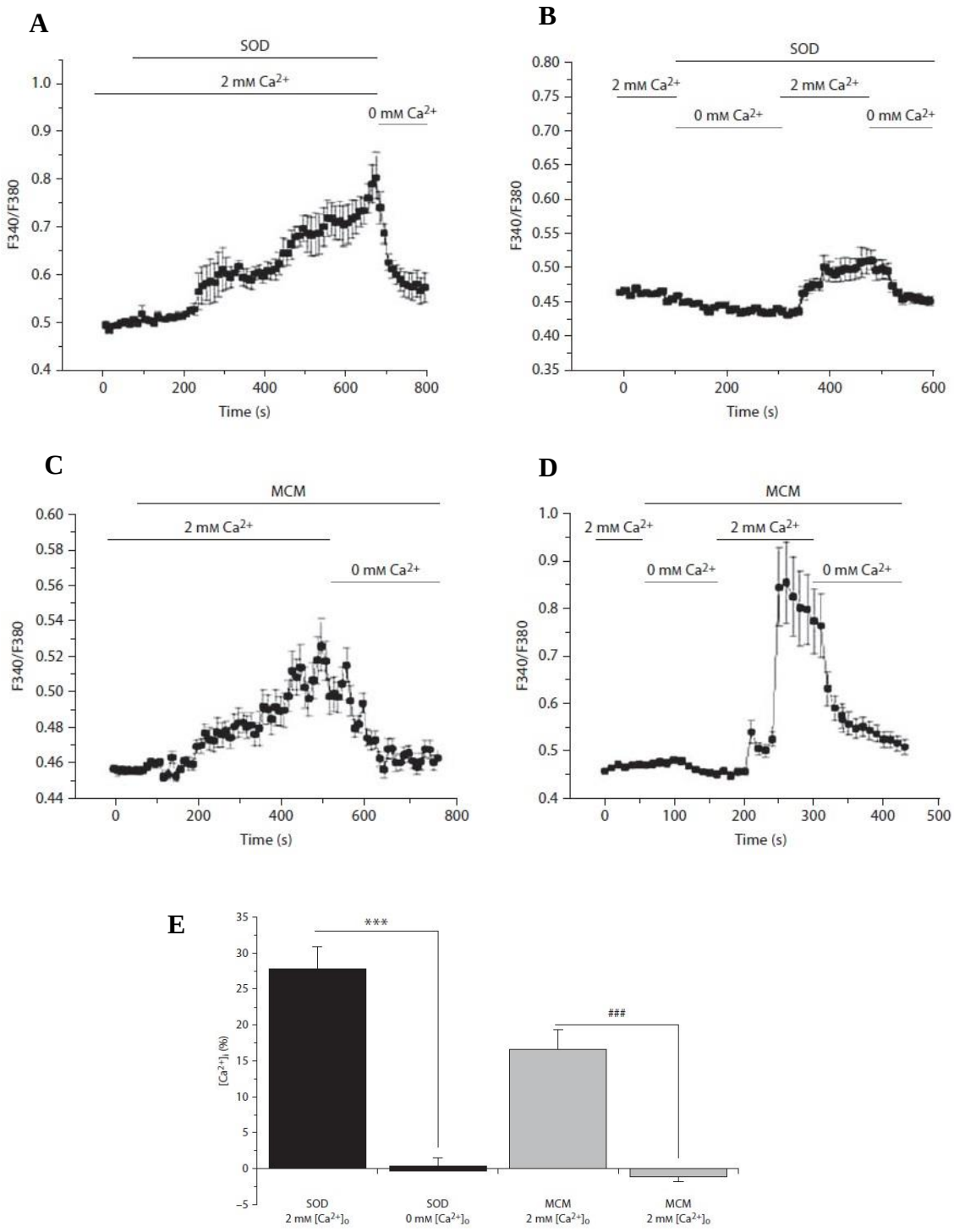

Fig. 12. SOD1 and MCM promote $[Ca^{2+}]i$ elevation in cultured CGNs. Representative variations in $[Ca^{2+}]i$ measured in fura-2- loaded cultured CGNs stimulated with 6.25 U/ml SOD1 in the presence (**A**) and absence of 2 mM $[Ca^{2+}]_o$ (**B**). Representative $[Ca^{2+}]_i$ traces measured upon MCM challenge in the presence (**C**) and absence of 2 mM $[Ca^{2+}]_o$ (**D**). The traces are representative of 10–15 cells of each experimental group studied in at least three different experimental sessions. (**E**) Histogram of the mean increase in maximal $[Ca^{2+}]_i$ rise with respect to the basal level upon exposure to 6.25 U/ml SOD1 and MCM with and without $[Ca^{2+}]_o$. The results are reported as intracellular calcium increases (% peak/basal). Data are expressed as the means ± SE of several cells in the various conditions. Triple marks indicate the statistical analysis performed with Student's t test. *** $p < 0.001$ compared the effect of SOD1 on $[Ca^{2+}]_i$ to $[Ca^{2+}]_o$ conditions; ### $p < 0.001$ compared the effect of MCM on $[Ca^{2+}]_i$ to $[Ca^{2+}]_o$ conditions. (from Polazzi, Mengoni et al., 2013).

Interestingly, administration of SOD1 at different concentrations, from 0.75 to 12.5 U/ml, was able to induce a dose-dependent increase in $[Ca^{2+}]_i$ (fig. 13A). In order to verify whether the effect of SOD1 on calcium dynamics depended on its catalytic activity, we performed a control experiment in which a SOD1 mimetic substance, MnTMPyP, possessing similar catalytic activity failed to affect the calcium dynamics (data not shown), similarly to what previously shown in neuroblastoma cells (Mondola et al., 2004). On the basis of these results, we supposed that if the calcium flux was important for neuroprotection, interfering with it could abrogate the neuroprotection provided by SOD1, either exogenously added or endogenously present in the conditioned medium. This was actually the case since the extracellular Ca^{2+} chelator EDTA, which has no effect on the Cu-Zn site of SOD1 (Ye and English, 2006), significantly reverted the neuroprotection mediated by exogenous SOD1 and by 48-hour MCM (fig. 13B).

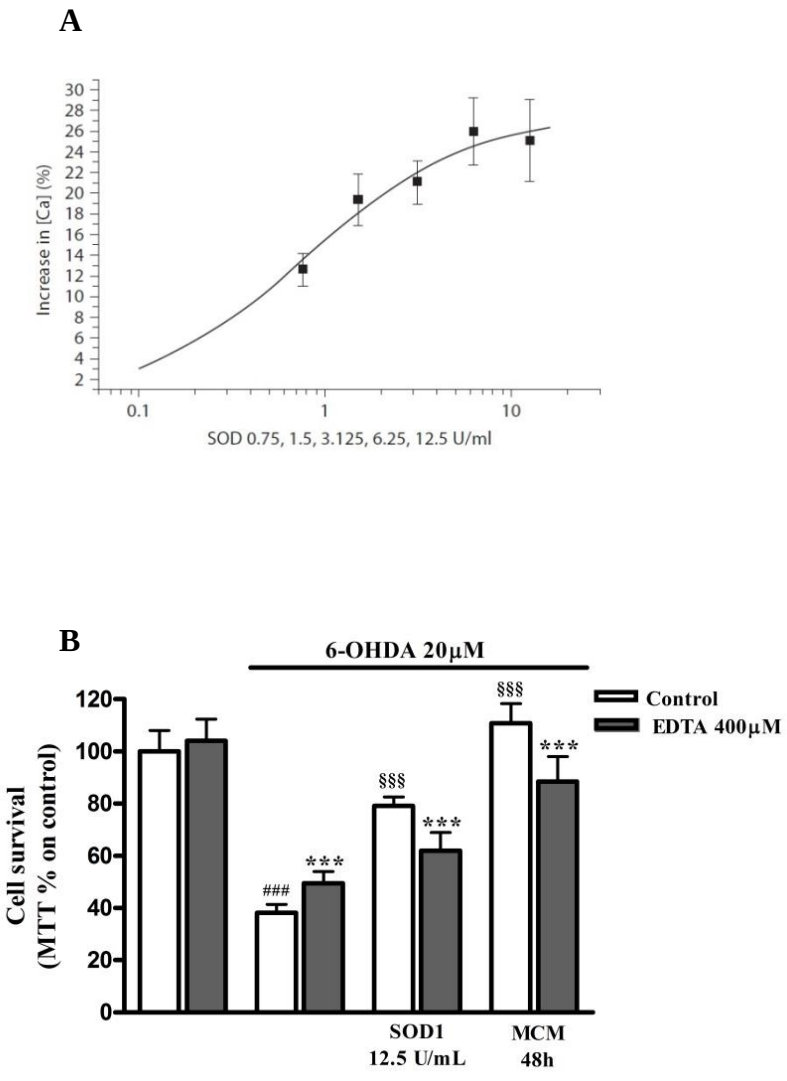

Fig.13. SOD1 promotes $[Ca^{2+}]i$ elevation in CGNs and the extracellular Ca^{2+} chelator EDTA decreases the neuroprotective effect of both MCM and SOD1 on 6-OHDA neurotoxicity. (**A**) Dose-dependent effect curve of SOD1 on $[Ca^{2+}]_i$ increase at different concentrations (0.75, 1.5, 3.125, 6.25, and 12.5 U/ml). The results are reported as intracellular calcium increases (% peak/ basal) and are representative of 25–30 cells of each experimental group studied in at least three different experimental sessions. The plot was fitted to the equation y = max·x/(x + k). (**B**) MTT assay on CGNs cultures treated for 24 h with 20 µM 6-OHDA in the presence or absence of exogenous SOD1 12.5 U/ml, 48 h MCM, and 400 µM EDTA. Bars are the means ± SE of 6–8 experiments; ### $p < 0.001$ compared to the control without 6-OHDA; §§§ $p < 0.001$ compared to the control with 6-OHDA; *** $p < 0.001$ compared to its own control with 6-OHDA. (from Polazzi, Mengoni et al., 2013).

4.1.9 ROLE OF SOD1 AGAINST OTHER FORMS OF NEUROTOXICITY

To investigate the specificity of SOD1 protection against different forms of neurotoxicity, we added recombinant SOD1 at high concentrations to CGNs exposed to two well-known and widely used models of neurotoxicity, i.e. a shift from high to low potassium medium or chronic exposure to glutamate. As previously demonstrated, 48-hour MCM significantly protected CGNs in both conditions (Polazzi et al., 2001; Eleuteri et al., 2008), but the addition of exogenous SOD1 to a non-conditioned medium had no significant neuroprotective effects (fig. 14A,B). These results confirmed that the neuroprotective effect of SOD1 was specific towards 6-OHDA-induced neurodegeneration and suggested that microglia-mediated neuroprotection could be exerted by multiple released factors with diverse effects on different types of neurodegeneration, further demonstrating the complexity of microglia-mediated neuroprotection.

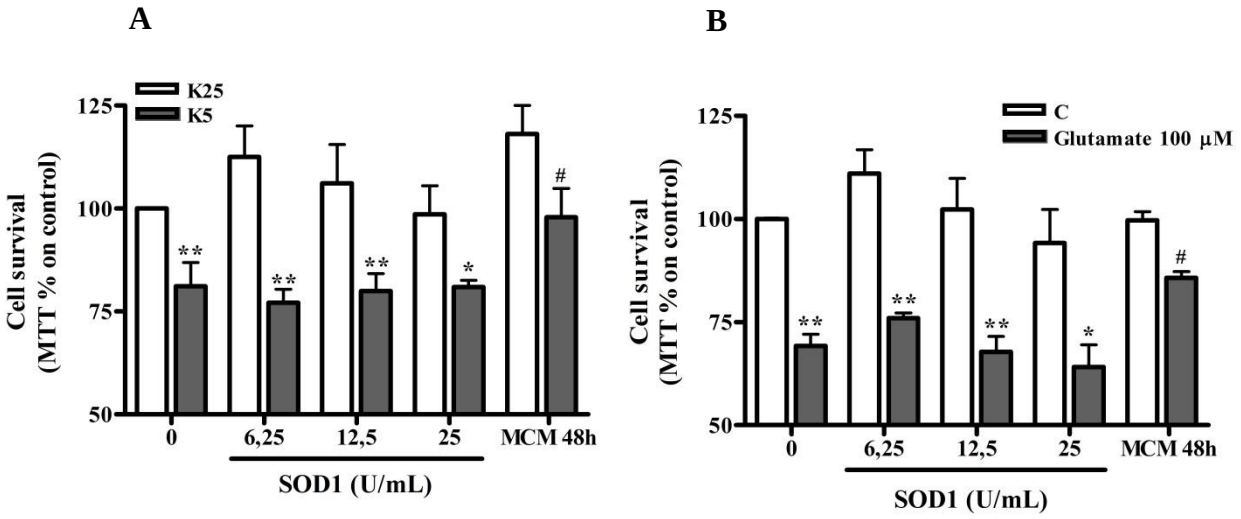

Fig. 14. Effect of exogenous SOD1 on different models of neurotoxicity induced in CGCs by a shift to low potassium and glutamate excitotoxycity. (**A**) Cell survival (MTT assay) of CGN cultures shifted to low potassium for 24 h in the presence or absence of different amounts of exogenous SOD1 (6.25, 12.5, and 25 U/ml) or of medium conditioned for 48 h by unstimulated microglial cell cultures (100% of the culture media). Bars are the means ± SE of four different experiments run in triplicate. * $p < 0.05$; ** $p < 0.01$ compared to its own control condition; # $p < 0.05$ compared to low potassium conditions (without SOD1). (**B**) Cell survival (MTT assay) of CGNs cultures treated for 24 h with 100 µM glutamate in the presence or absence of different amounts of exogenous SOD1 (6.25, 12.5, and 25 U/ml) and of medium conditioned for 48 h by unstimulated microglial cell cultures (100% of the culture media). Bars are the means ± SE of four different experiments run in triplicate. * $p < 0.05$; ** $p < 0.01$ compared to its own control condition; # $p < 0.05$ compared to glutamate conditions (without SOD1). Bonferroni's test after ANOVA. (from Polazzi, Mengoni et al., 2013).

Important note:

most of the results described in this part have been published as:

Polazzi E*, Mengoni I*, Caprini M, Pena-Altamira 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 (*co-first autors).

4.2

Evidences on microglial ApoE secretion, neuroprotection and neuronal modulation of the release

4.2.1 MICROGLIAL APOE SECRETION

We have previously demonstrated that rat primary microglial cells release factors, which are neuroprotective towards CGNs exposed to various neurodegenerative stimuli (Polazzi et al., 2001; Polazzi et al., 2009). By using a proteomics approach (Polazzi et al., 2013), we identified in MCM samples several protein spots, among which ApoE was one of the most expressed, together with two 19 and 17 kDa ApoE fragments, suggesting its proteolytic fragmentation (Fig. 9 introduction). In order to characterize ApoE release in our microglial primary cultures, we performed western blot analysis of different MCM. We observed that microglia constitutively secreted ApoE, which progressively accumulated in the medium, being detectable after 2 h, increasing from 8 h to 12 h and peaking from 24 h to 48 h of microglial conditioning (fig.15A). As we did for SOD1, we quantified the amount of secreted ApoE in neuroprotective 48h-MCM, comparing the intensity of bands derived from known volumes of 48h-MCM (i.e. 500µl and 250µl) with those obtained from known amounts of recombinant human ApoE2 (from 10 to 100pmol), by using the calibration curve method. ApoE concentration in 48h-MCM was estimated to be 138 pmol/ml.

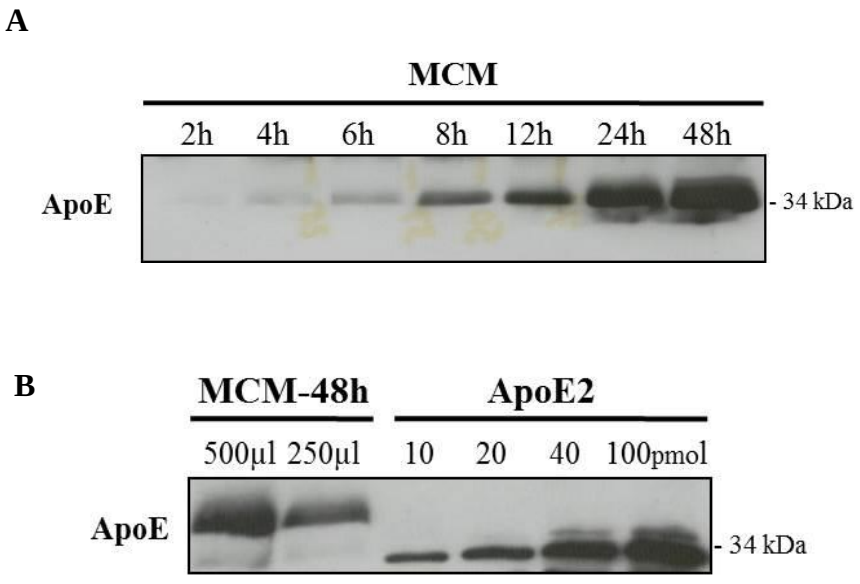

Fig. 15. ApoE is constitutively released by microglia. Representative Western blot analyses. (**A**) ApoE protein expression in MCM from 2 h to 48 h (500µl MCM) shows time-dependent ApoE release and accumulation in MCM. (**B**) ApoE protein expression in 48h-MCM (500µl and 250µl) compared to increasing defined amounts of exogenous human ApoE2 (from 10 to 100 pmol). Subsequent densitometric quantification allowed quantitative evaluation of ApoE released in neuroprotective 48h-MCM, which is 138 pmol/ml.

4.2.2 INSIGHTS ON THE PATHWAY INVOLVED IN APOE SECRETION

Considering that in macrophages and astrocytes ApoE is constitutively secreted by the classical ER/Golgi-dependent secretory pathway (Dekroon and Armati, 2001; Kockx et al., 2008), we investigated whether this pathway was also involved in microglial ApoE secretion. To this aim, we exposed microglial cells to Brefeldin A, an inhibitor of Golgi-dependent protein secretion, which acts by disassembling this apparatus (Klausner et al., 1992). Treatment of microglia with 5 µg/mL BFA for 4 h resulted in a complete absence of ApoE in MCM analyzed by Western blot analysis (Fig. 16A, upper lane) due to blocked release and not to synthesis interference, since ApoE accumulated in corresponding cellular lysates (Fig. 16A, lower lane), clearly suggesting intracellular retention of synthesized ApoE protein. Confirming evidence derived from immunofluorescence experiments (Fig. 16B). In particular, in control conditions ApoE immunoreactivity showed a supra-nuclear localization corresponding to normal configuration of Golgi apparatus, whereas in BFA-stimulated cells ApoE-staining was widespread, indicating Golgi disorganization and dispersion throughout the cytoplasm.

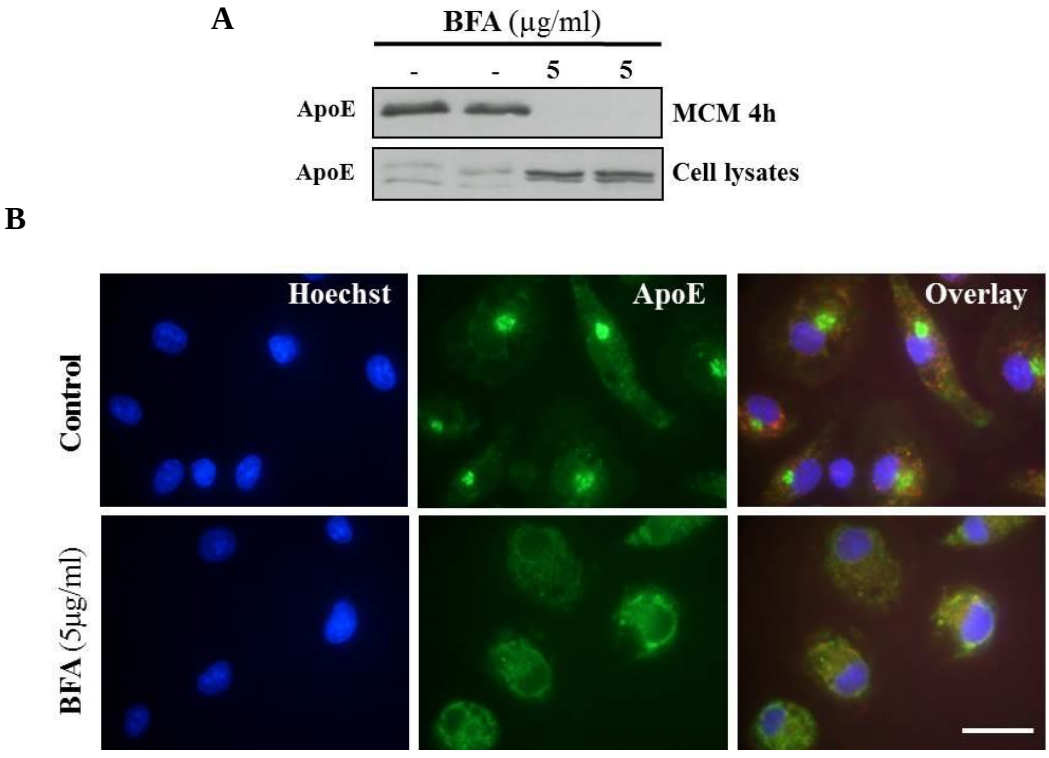

Fig. 16. ApoE is released by microglia through the classical ER-Golgi secretion pathway (**A**) representative western blot analysis shows that stimulation for 4 h with 5 µg/mL Brefeldin A causes a marked reduction of ApoE release in treated MCM, compared to controls, and accumulation of ApoE in corresponding BFA-treated microglial cells. (**B**) Immunocytochemical analysis of ApoE (FITC labeled, green) and chromatin staining (Hoechst, blue) in microglial cells treated for 4 h with 0.5 µg/mL BFA (lower figures) and corresponding controls (upper figures) further supports the role of the ER-Golgi-pathway in microglial ApoE release. Scale bar: 15 µm.

4.2.3 MODULATION OF APOE SECRETION BY MICROGLIAL ACTIVATION

We then tested whether constitutive release of ApoE by microglia was altered in different activation conditions, a common state in brain pathology (Czirr and Wyss-Coray, 2012). Microglia stimulation for 24 h with increasing concentrations of the most commonly used microglial activator, LPS (10-1000 ng/ml), significantly decreased ApoE levels both in the medium and cell lysates (Fig. 17A), confirming a negative regulation of both ApoE synthesis and release by microglial activation, as previously reported (Saura et al., 2003; Mori et al., 2004).

We also tested whether activation of P2X7 receptor, associated with cellular damage and chronic brain inflammation (Weisman et al., 2012), could negatively modulate ApoE release, similarly to LPS. P2X7 receptor is a member of the purinergic P2X family of ATP-gated ion channels and high levels of extracellular ATP are required for its rapid activation. As with LPS, stimulation for 4 h with increasing concentrations (50-200 µM) of Benzoyl-ATP (a specific agonist of P2X7 receptor, more stable than ATP) resulted in significant reduction of released ApoE, without any clear effect on intracellular ApoE levels (Fig.17C).

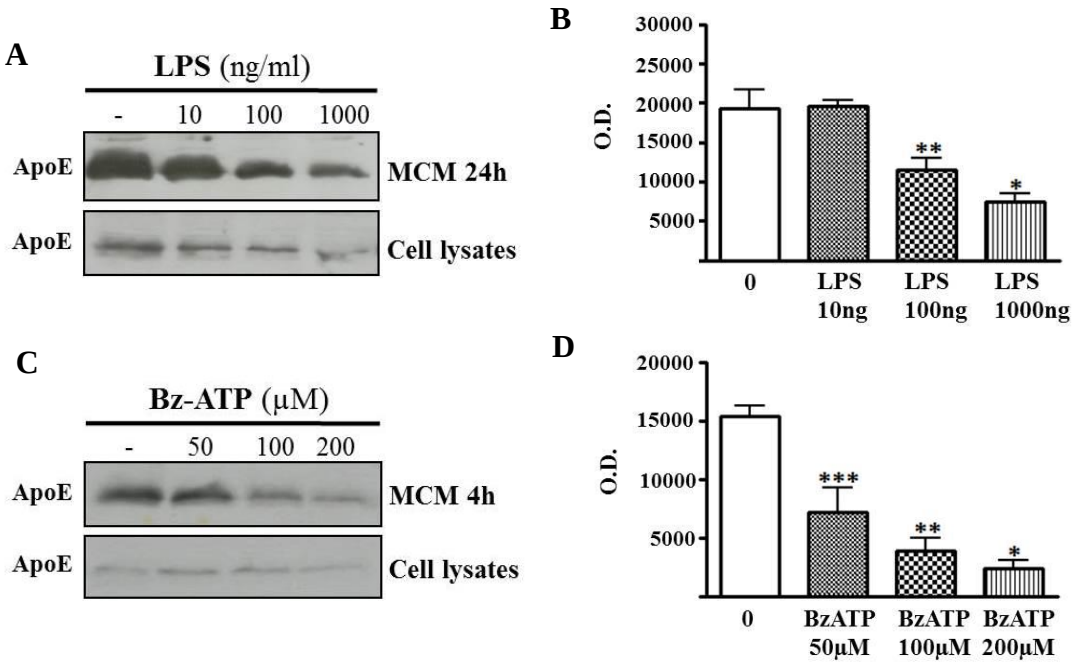

Fig. 17. Inflammatory stimuli down regulate microglial constitutive ApoE release. Microglial stimulation for 24 h with increasing concentrations of LPS, results in significant decrease of ApoE secretion, as demonstrated by western blot analysis (**A**) and relative densitometries (**B**). 4h treatment of microglial cells with increasing concentration of Bz-ATP, determines a decrease in ApoE release, as shown by Western blot analysis (**C**) and relative densitometries (**D**). All western blots shown are the representative analysis of at least three experiments and each graph bar is the mean ± SE of three different experiments. ***p<0,001; **p<0,01; *p<0,05 compared to control conditions, Student's t test.

Considering that neurons influence microglial activation states, as well as the microglial secretory pathway (as previously described in the introduction), we decided to study whether neurons could modulate microglial ApoE release in unstimulated or activated conditions. To this aim, we treated microglial cells for 24 h with medium previously conditioned for 24 h by mature CGNs (CGNm), thus testing, by Western blot analysis, ApoE release from neuronal-stimulated microglia. As shown in figure 18A, CGNm induced much higher ApoE release levels from microglia, compared to control medium. Moreover, the increased ApoE accumulation was not due to neuronal release, since ApoE was not detectable in a medium conditioned for 24 hours by cerebellar granule neurons (CGNm(c)), used as control.

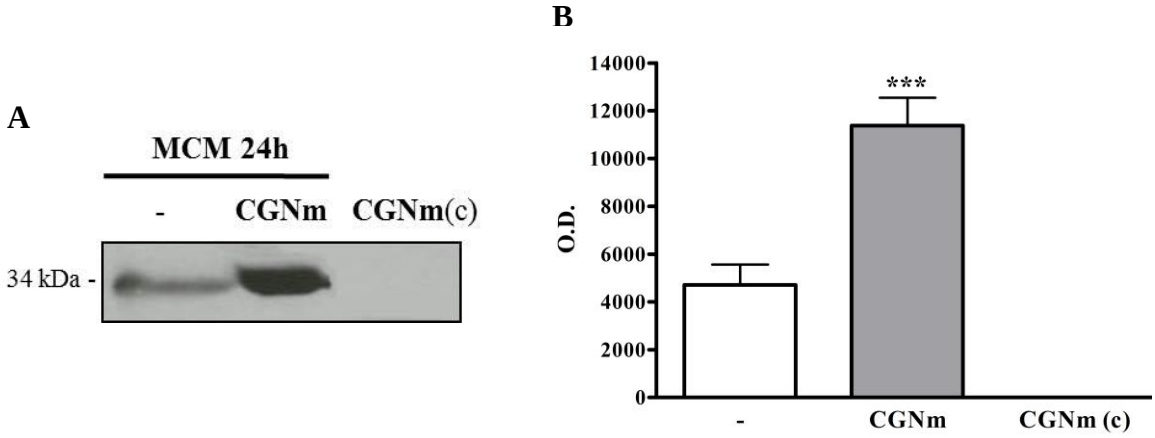

Fig. 18. CGNs potentiate constitutive ApoE release by microglia (**A**) Western blot analysis and (**B**) corresponding densitometries of media conditioned for 24 h by control microglia (-) or microglia stimulated with medium previously conditioned for 24 h by mature CGNs (CGNm). CGNm(c) represents control medium conditioned for 24 h by CGNs. Graph bar are the mean ± SE of three different experiments; ***p<0,001 compared to control condition; Student's t test.

This suggests that CGNs release in the medium factors able to stimulate additional ApoE release from microglia. Neuronal-stimulated microglial ApoE release appeared to involve the same intracellular constitutive Golgi-dependent pathway, since 5 µg/mL BFA blocked ApoE accumulation in medium from microglia exposed for 4h to 24h-CGNm (Fig. 19A). Moreover, different states of microglial activation, i.e. stimulated by LPS or Bz-ATP, differently regulated microglial ApoE release induced by 24h-CGNm. In fact, LPS treatment (100 ng/mL) for 24h decreased ApoE microglial release induced by CGNm (Fig.19C), similarly to pure microglial cultures. On the other hand, activation of microglial P2X7 receptor by 4h stimulation with 100 µM Bz-ATP (Fig. 19E) did not result in any significant reduction of microglial-ApoE release induced by CGNm, suggesting the

existence of a delicate regulatory balance for microglial ApoE secretion from different microglia-activating stimuli.

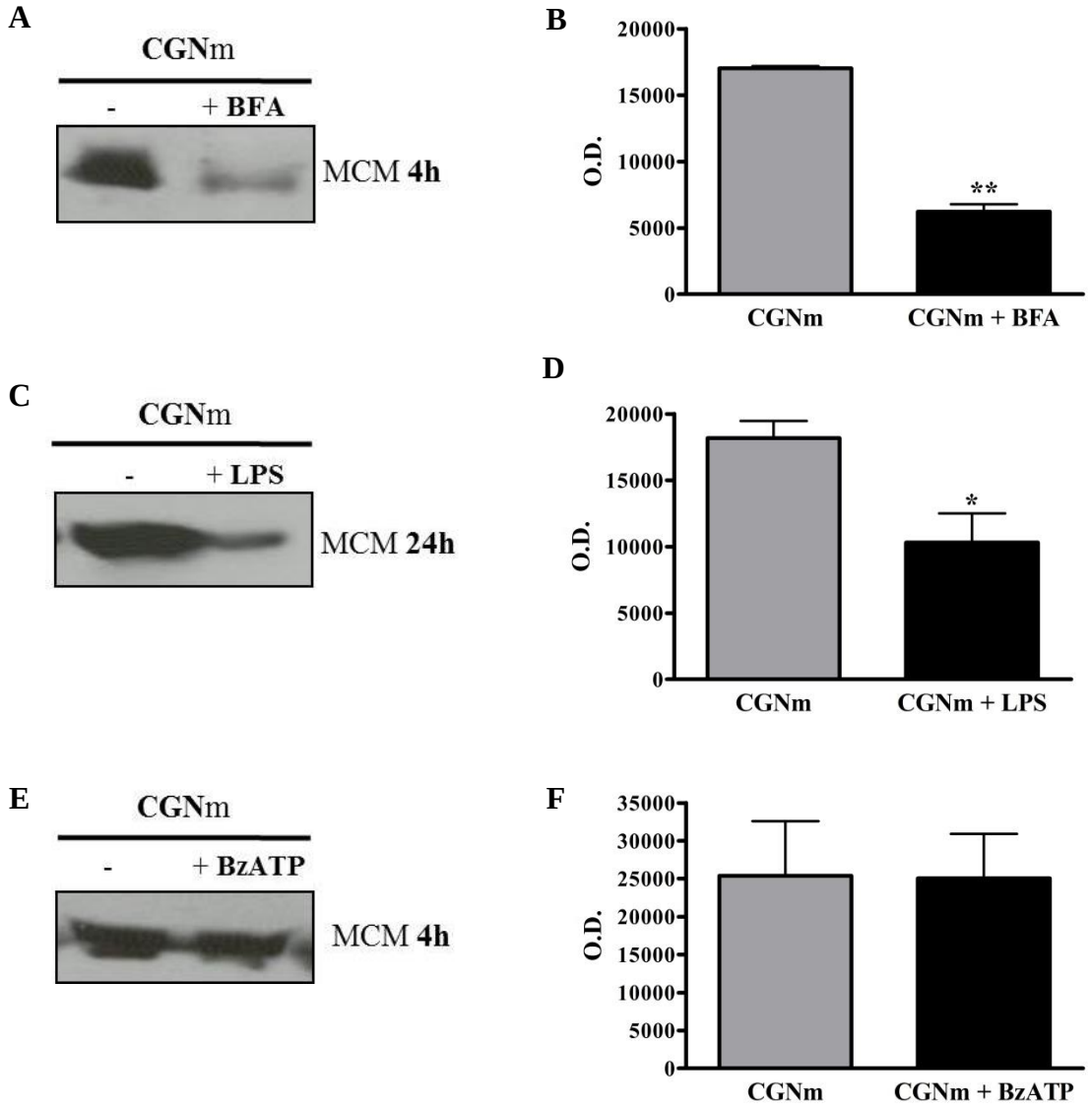

Fig. 19. Neuronal-stimulated microglial ApoE release is differentially down-regulated by diverse inflammatory stimuli. Four hour treatment with 0.5 μg/mL BFA decreases neuronal-stimulated microglial ApoE release, as shown by Western blot analysis (**A**) and relative densitometries (**B**). The same type of analysis demonstrate that microglial activation through exposure for 24 h to 100 ng/mL LPS results in decreased microglial ApoE secretion stimulated by CGNm, compared to conditioned medium from LPS-untreated, neuronal-stimulated cells (**C-D**), while stimulation for 4 h with 100 μM Bz-ATP does not determine any significant change in neuronal-stimulated microglial ApoE secretion (**E-F**). All Western blots shown are the representative analysis for each type of experiment and each graph bar is the mean ± S.E. of three different experiments; ***p<0,001 compared to control condition; **p<0,01; *p<0,05 compared to control CGNm-stimulated MCM, Student's t test.

4.2.4 NEURONAL MODULATION OF MICROGLIAL APOE SECRETION IN A PATHOLOGICAL CONDITION

In order to further explore differences in neuronal stimulation of microglial ApoE release not only in physiological, but also pathological conditions, we compared ApoE release from microglia treated with medium conditioned for 24 h by CGNs either exposed to 20 µM 6-OHDA or in control conditions. Previous data (Polazzi et al., 2009) showed that exposure to 6-OHDA resulted in an extensive degeneration of CGNs. Medium conditioned by CGNs exposed to 6-OHDA exerted a stimulatory effect on microglial ApoE release very similar to the medium from healthy neurons.

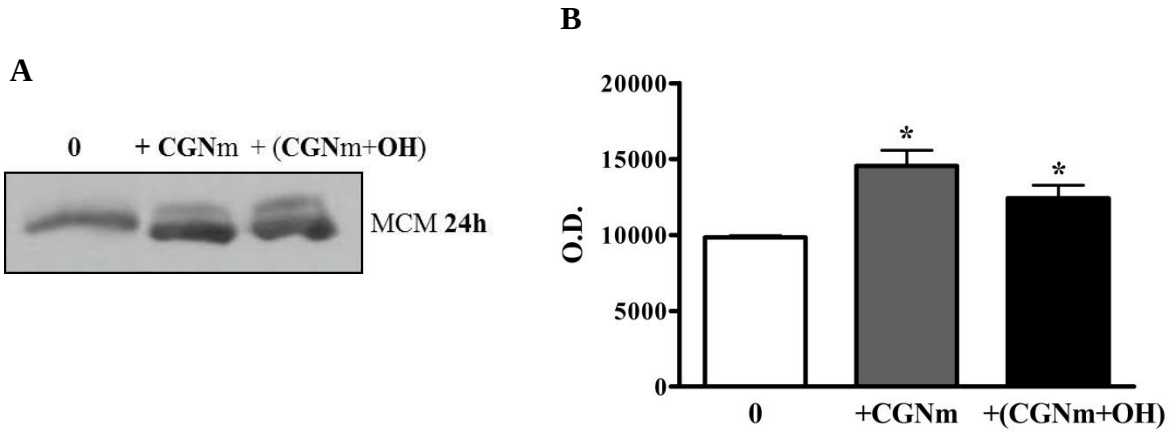

Fig. 20. Microglia-neuron physical contact down regulates microglial ApoE secretion (**A**) Western blot analysis and (**B**) relative densitometries of media conditioned for 24 h by microglia stimulated with medium previously conditioned for 24 h by CGNs (CGNm) in physiological or neurodegenerative conditions, i.e. following exposure to 20 µM 6-OHDA (CGNm+OH), do not show any significant change in neuronal-stimulated microglial ApoE release. Each graph bar is the mean ± S.E. of three different experiments; *p<0,05; Student's t test.

We also used microglia-neurons co-cultures in order to investigate reciprocal interactions between these cell types, mediated not only by exchange of released molecular signals, but also by physical cell contact (Polazzi et al., 2001; 2009). Therefore we studied co-cultures of primary microglia and CGNs in physiological conditions and in presence of 20 µM 6-OHDA. We observed a strong induction of microglial ApoE release in co-cultures with healthy CGNs, thus confirming previous results obtained with CGNm. On the other hand, there was a significant decrease in microglial ApoE release in neurodegenerative conditions, i.e. when co-cultures were exposed to 20 µM 6-OHDA (Fig. 21 A-B).

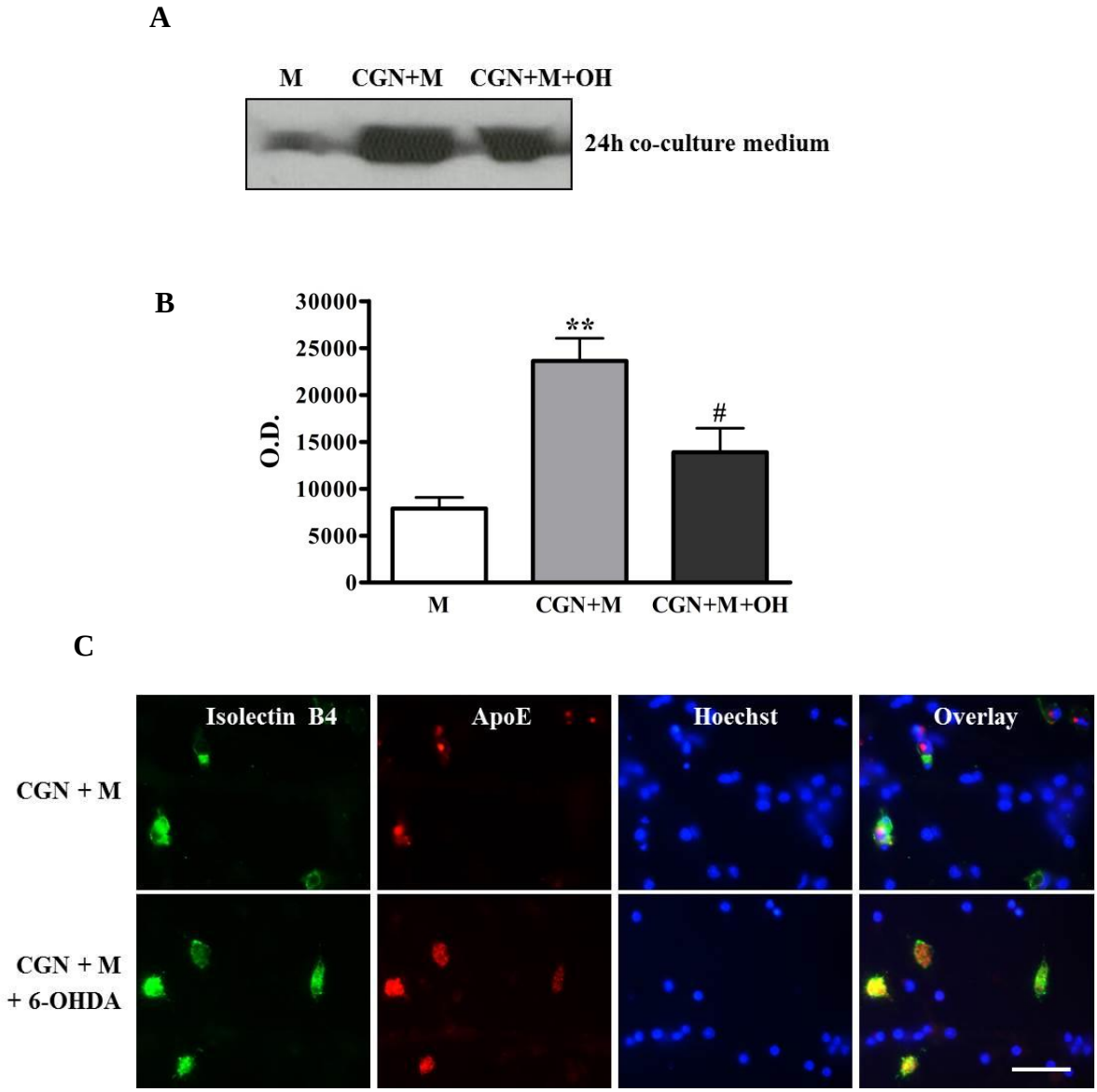

Fig. 21. ApoE secretion is significantly reduced by 6-OHDA neurotoxicity compared to controls in co-cultures (**A**) Representative Western blot analysis with corresponding densitometries (**B**) of media conditioned by CGNs-microglia co-cultures, with cells in physical contact, in control conditions (M+CGN) or in presence of 20 µM 6-OHDA for 24 h (M+CGN+OH). Each graph bar is the mean ± S.E. of three different experiments; **p<0,01 compared to control condition; $^{\#}$p<0,05 Microglia+CGN compared to Microglia+CGN+OH; Student's t test. (**C**) Immunocytochemical analysis of ApoE (TRITC labeled, red) and microglial staining (Isolectin B_4, green) in CGNs-microglia co-cultures treated with 20 µM 6-OHDA and in corresponding controls. (scale bar: 30 µm).

Decreased ApoE release in these conditions could not be attributed to microglial damage or neuronal death, since 6-OHDA did not affect microglial survival and microglia are neuroprotective towards CGNs (Polazzi et al., 2009). However, co-culture affected ApoE localization in microglia. In physiological conditions, immunoreactivity of microglial ApoE in co-cultures with CGNs showed a typical localization corresponding to normal

configuration of Golgi apparatus, whereas in presence of 20μM 6-OHDA, microglial ApoE-staining was widespread, with Golgi disorganization and dispersion throughout the cytoplasm, similarly to what observed after BFA treatment, which leads to decreased microglial ApoE release (Fig. 21C). The different intracellular localization of microglial ApoE between physiological and pathological conditions could explain the decreased ApoE accumulation in the medium from 6-OHDA treated co-cultures compared to controls.

4.2.5 APOE CONTRIBUTION TO MICROGLIAL NEUROPROTECTION

We explored the contribution of ApoE to microglial neuroprotection in co-culture conditions. We used this co-culture model to demonstrate, through ApoE silencing, that ApoE production from microglial cells contributes to their neuroprotective action. Preliminarily, we observed that ApoE silencing in microglia with specific siRNA resulted in decreased ApoE release, while Negative Control siRNA had no effect (Fig. 22A). Moreover, in co-cultures, microglia ApoE silencing abrogated the neuroprotection granted to CGNs challenged with neurotoxic 6-OHDA (Fig. 22B).

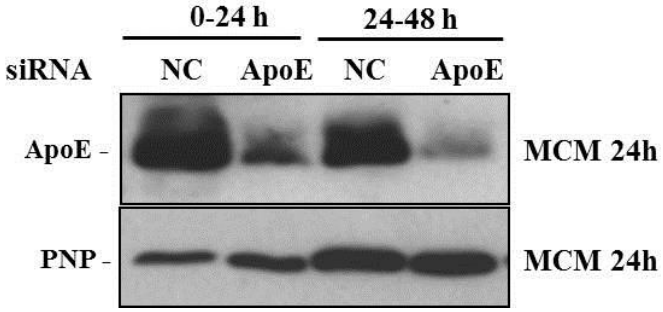

Fig.22. Reduced microglial ApoE secretion obtained through siRNA mediated silencing. Western blot analysis of Apo E release in media from primary rat microglial cells trasfected with siRNA-ApoE, conditioned for the first 24 hours(0-24) or the following 24 hours (24-48) after transfection, compared to MCM from cultures trasfected with siRNA-Negative Control, for the same time frames (upper panel). The specificity of siRNA used was demonstrated by the unchanged amount of released Purine Nucleoside Phosphorylase (PNP), an enzyme we know to be released by microglial cells (our unpublished data), used as control.

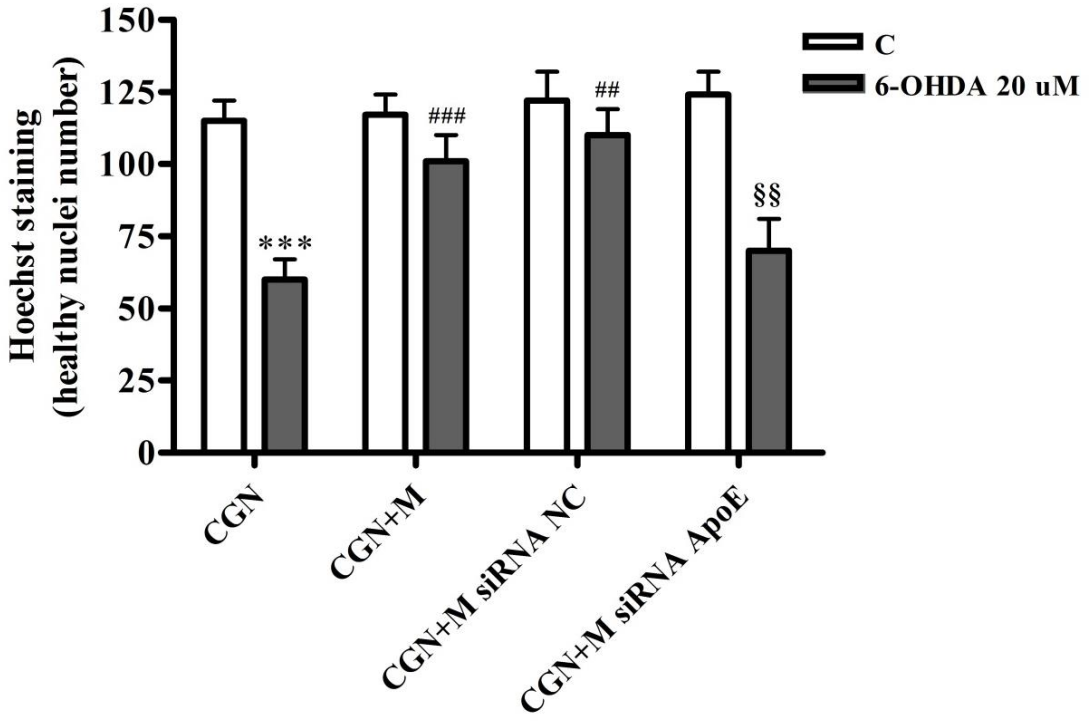

Fig. 23 Neuroprotective action of microglia in co-cultures is decreased by siRNA mediated ApoE silencing in microglia. Normal nuclei counting: microglial cells transfected with a siRNA Negative Control (NC) completely protect CGNs from 6-OHDA neurotoxicity, whereas microglial cells in which ApoE is knocked down, do not exert any significant neuroprotective action. Each point is the mean ± S.E. of 3 different experiments run in triplicate; **p<0,01 compared to control CGNs; $^{###}$p<0.001, compared to 6-OHDA treated CGNs, Student's t test.

To further confirm ApoE-mediated neuroprotection, exogenous ApoE was added to non-conditioned serum-free medium to observe whether this could partially replicate the neuroprotective effect of MCM, in which ApoE accumulates. To this aim, we exposed mature CGNs (7DIV) for 24 h to serum-free medium with or without 20 μM 6-OHDA and different concentrations of exogenous ApoE, i.e. human ApoE2, which closely resembles rat ApoE and has been previously shown to be neuroprotective in different neuropathology models (Hatters et al., 2006; Holtzman et al., 2012). As with MCM, exogenously added ApoE was significantly neuroprotective towards 6-OHDA neurotoxicity in a dose-dependent way.

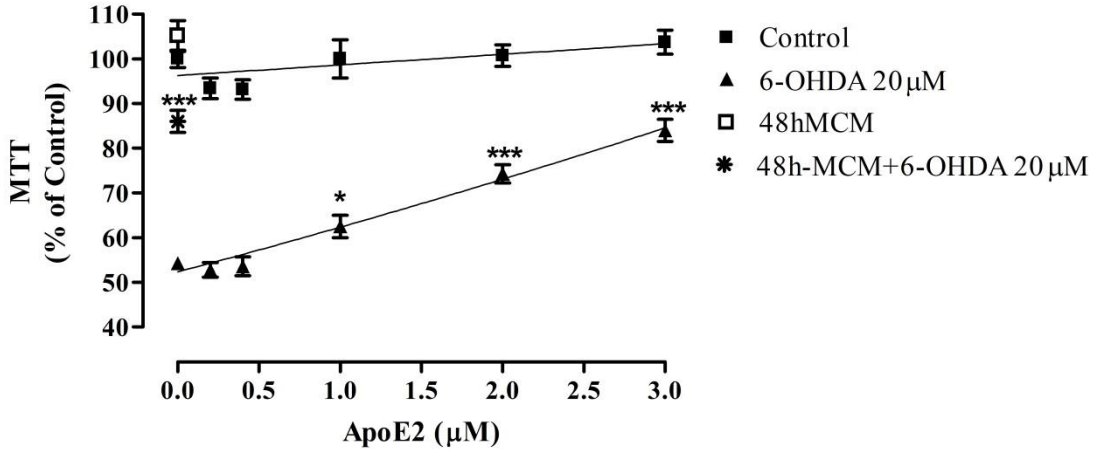

Fig. 24. Neuroprotective action of exogenous ApoE on damaged neurons. MTT assay of control CGNs or CGNs treated with 20 μM 6-OHDA for 24 h in the presence or absence of exogenous ApoE increasing concentrations (human ApoE2) or of 48 h-MCM show that exogenous ApoE2 is neuroprotective towards CGNs exposed to 20 μM 6-OHDA for 24 h, similarly to 48h-MCM. Each point is the mean ± S.E. of four different experiments run in triplicate; *p<0.05, ***p<0,001 compared to 20 μM 6-OHDA exposure in absence of exogenous ApoE.

4.3

Comparison of microglial M1/M2 phenotype in wild type and transgenic rat model of the Alzheimer's like amyloid pathology

4.3.1 MICROGLIAL PHENOTYPE IN PRE-PLAQUE STAGE OF A RAT MODEL OF ALZHEIMER'S DISEASE

Neurodegenerative processes lead to the activation of microglial cells, which may acutely serve a beneficial purpose, but when chronically activated they further promote and exacerbate neurodegeneration (Heneka et al., 2010).

In a model of Alzheimer's disease, we described how adult microglia can be isolated from the McGill-R-Thy1-APP transgenic rat and we performed a study to delineate their profile in this neurological disorder. In order to obtain a better characterization of the phenotype, we analyzed microglial cells harvested from three different areas: cortex, hippocampus and cerebellum. The latter was used as an internal "control" region, as the cerebellum is usually less compromised by the pathology if compared to the other two areas. Importantly, the cells were freshly extracted from the brain and were not cultured prior to the analysis, and mRNAs were extracted from the pellets obtained at the end of the microglial isolation protocols.

The study involved two groups of animals, composed by rats of 5 or alternatively 16-17 months of age, representing an initial and an advanced stage of the pathology, respectively. Within each group the microglia from 4 wild type rats was compared to the ones harvested from 4 McGill-R-Thy1-APP transgenic rats. From each animal, microglia isolated from cortex, hippocampus and cerebellum were kept separate during all the steps (from cell isolation to qRT-PCR analysis).

First of all we analyzed the levels of some control markers, i.e. Ionized calcium binding adaptor molecule 1 (Iba1) for microglia, glial fibrillary acidic protein (GFAP) for astrocytes and neuron-specific nuclear protein (NeuN) for neurons. None of them showed any significant difference between cell preparation from wild type and transgenic animals (not shown).

Then we started with the analysis of the makers usually considered to characterize the M1 microglial phenotype. In particular we choose: interleukin-6 (**IL-6**), interleukin-1beta (**IL-1β**), inducible Nitric Oxide Synthase (**iNOS**), Cicloxigenase-2 (**COX2**), interleukin-2 (**IL-2**), interleukin-12alpha (**IL-12α**), interleukin-23 alpha (**IL-23α**), Macrophage inflammatory protein 1 alpha (**MIP1α**) and Tumor Necrosis Factor alpha (**TNFα**).

From the analysis at 5 months of age, only two differences resulted significant: **IL-23α** and **IL-12α** were upregulated in transgenic rats (compared to wild type rats) in hippocampal and cerebellar microglia, respectively.

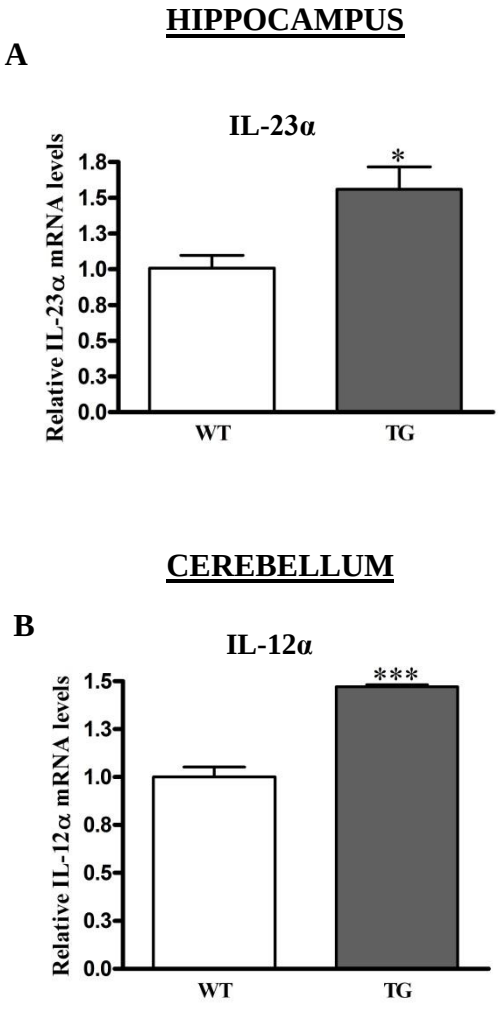

Fig. 25. Different microglial M1 marker expression in 5 months old rats. Quantitative real-time PCR analysis revealed significantly higher Il-23α and Il-12α mRNA levels in (**A**) hippocampal and (**B**) cerebellar microglia, respectively. Il-23a and Il-12a mRNA expression was normalized to the housekeeping gene β-actin. Error bars represent mean ± SE. *P < 0.05; **P< 0.01; Student's t-test.

After that we proceeded with the analysis of the M2 markers. In this case we designed primers for the following genes:

Interleukin-10 (**IL-10**), chitinase 3-like-3 (CHI3L3, also called **YM1**), Arginase-1 (**Arg1**), Mannose receptor c, type 1 (**Mrc1**), transforming grow factor, beta 1 (**TGF-β1**) and triggering receptor expressed on myeloid cells 2 (**TREM2**).

Interestingly, in cortex and cerebellum M2 markers resulted downregulated in the microglia from transgenic rats, when compared to wild type.

The situation was opposite when considering the hippocampal microglia.

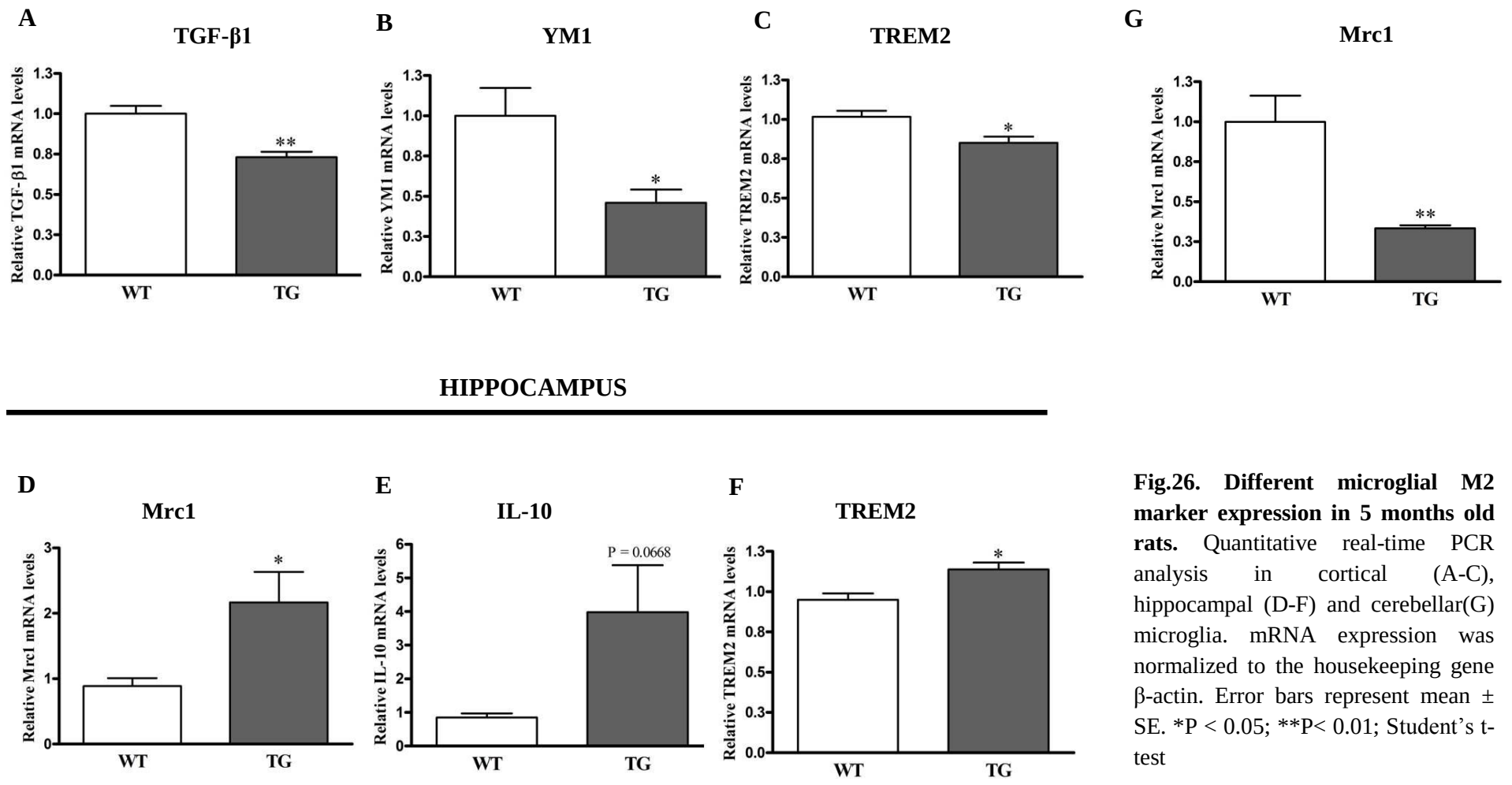

Fig.26. Different microglial M2 marker expression in 5 months old rats. Quantitative real-time PCR analysis in cortical (A-C), hippocampal (D-F) and cerebellar(G) microglia. mRNA expression was normalized to the housekeeping gene β-actin. Error bars represent mean ± SE. *P < 0.05; **P< 0.01; Student's t-test

4.3.2 MICROGLIAL PHENOTYPE IN POST-PLAQUE STAGE OF ALZHEIMER'S DISEASE

The same scheme of experiments was performed using microglial cDNA from older animals. In particular we used animals of 16-17 months of age, that are considered to mimic an advanced stage of the pathology in transgenic rats (Leon et al., 2010).

In general, the analysis revealed less variation compared to the previous study, where younger animals were involved. In fact, considering all the M1 markers analyzed, only for **iNOS** there was a significant difference in expression levels between wild type and transgenic microglia, in both cortex and hippocampus.

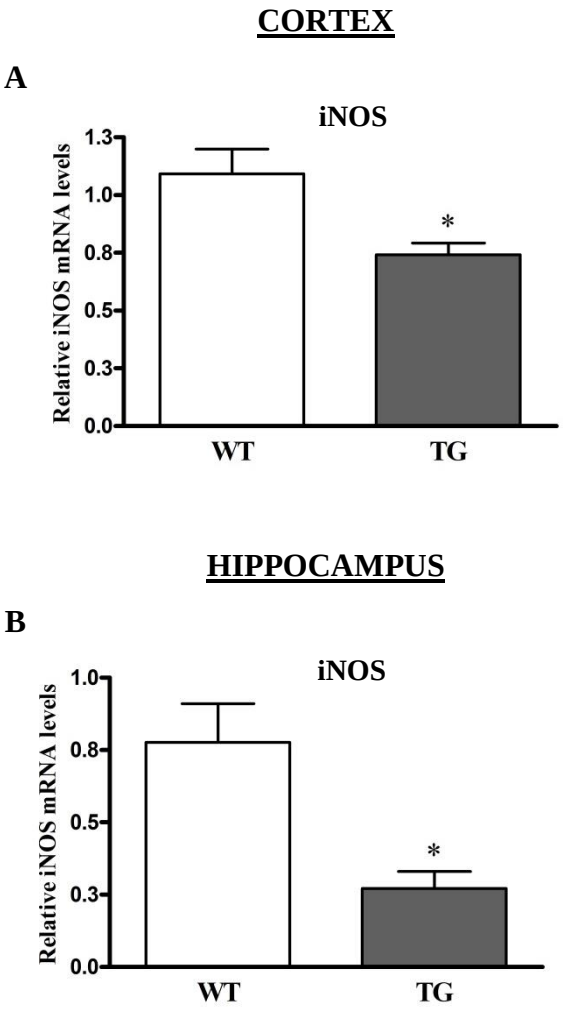

Fig. 27. Different microglial M1 marker expression in 16-17 months old rats. Quantitative real-time PCR analysis revealed significantly lower iNOS mRNA levels in (**A**) cortical and (**B**) hippocampal microglia, respectively. iNOS mRNA expression was normalized to the housekeeping gene β-actin. Error bars represent mean ± SE. *P < 0.05; Student's t-test.

The situation was not very different when the M2 markers were analyzed. In this case, only **TGFβ1** was down regulated in the hippocampal microglia of transgenic rats. The same trend was observed also for **YM1**, always in the hippocampus, in which the down regulation was almost reaching significance.

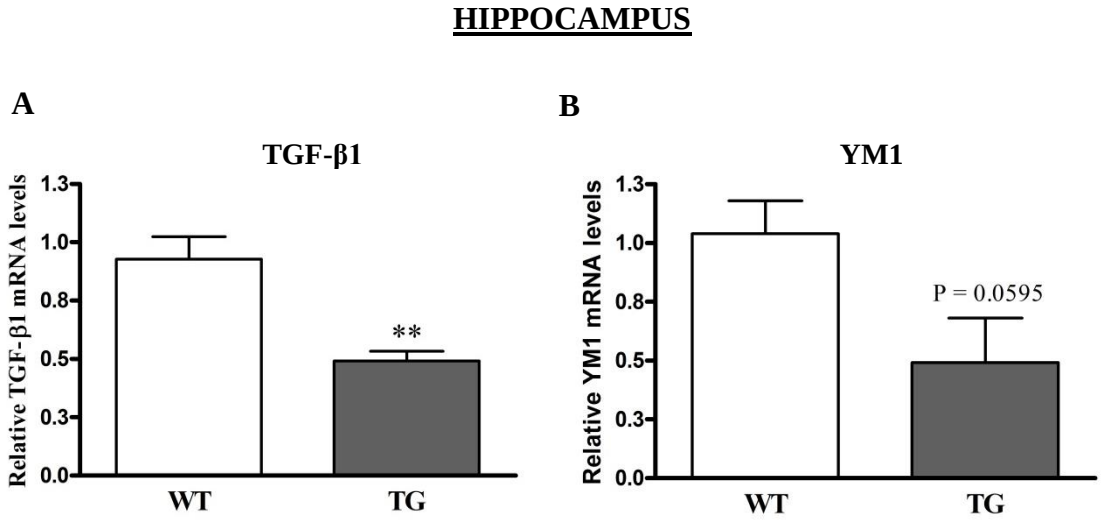

Fig. 28. Different microglial M2 marker expression in 16-17 months old rats. Quantitative real-time PCR analysis revealed significantly lower TGFβ1 mRNA levels (**A**) in the transgenic hippocampal microglia. The reported variation for YM1 (**B**) is almost significant (P=0.0595). mRNA expression was normalized to the housekeeping gene β-actin. Error bars represent mean ± SE. **P < 0.01; Student's t-test.

4.3.3 APOE AND LRP1 mRNA LEVELS

Considering the importance of ApoE as a risk factor for Alzheimer's disease, we investigated what happened to its mRNA levels in our samples.

Notably, we observed significant variations only in the young group of animals: a down regulation of microglial ApoE mRNA levels in the cortex and an up-regulation in the cerebellum, always considering transgenic compared to controls (Fig. 29 upper lane). No differences were observed in the group of older rats (Fig. 29 lower lane).

The same analysis has been performed for the low density lipoprotein receptor-related protein 1 (Lrp1).

Cellular uptake of ApoE is mediated by the low-density lipoprotein (LDL) receptor family that includes the low-density lipoprotein receptor (LDLR), very-low density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (ApoEr2), and the LDL receptor-related protein-1 (LRP1). Neuronal receptors (ApoEr2, VLDLR, and LRP1) have been implicated in neurite outgrowth, calcium homeostasis, kinase activation and cell migration (Beffert et al., 2004). A different subset of receptors is expressed on astrocytes and microglia: LDLR, LRP1, and VLDLR (Rebeck et al., 1993; Christie et al., 1996). ApoE signaling through these receptors involves mitogen-activated protein kinase (MAPK) pathways in neurons and glia (Hoe et al., 2006; Pocivavsek et al., 2009a; Pocivavsek et al., 2009b).

Importantly, LRP1 also interacts with the neuronally expressed amyloid precursor protein (APP) and regulates its proteolytical processing and the production of the Aβ peptide, a process that is of central importance for the pathogenesis of Alzheimer's disease (May et al., 2004). For this reason we considered this receptor as an interesting link between ApoE and Alzheimer's disease pathology.

Considering LRP1 mRNA levels we didn't observe any differences between wild type and transgenic microglia in both groups (Fig.30). The only exception was restricted to the hippocampal microglia of the rats of the 16-17 months group (Fig. 30E), where in the transgenic there was an almost significant down regulation of LRP1 mRNA levels.

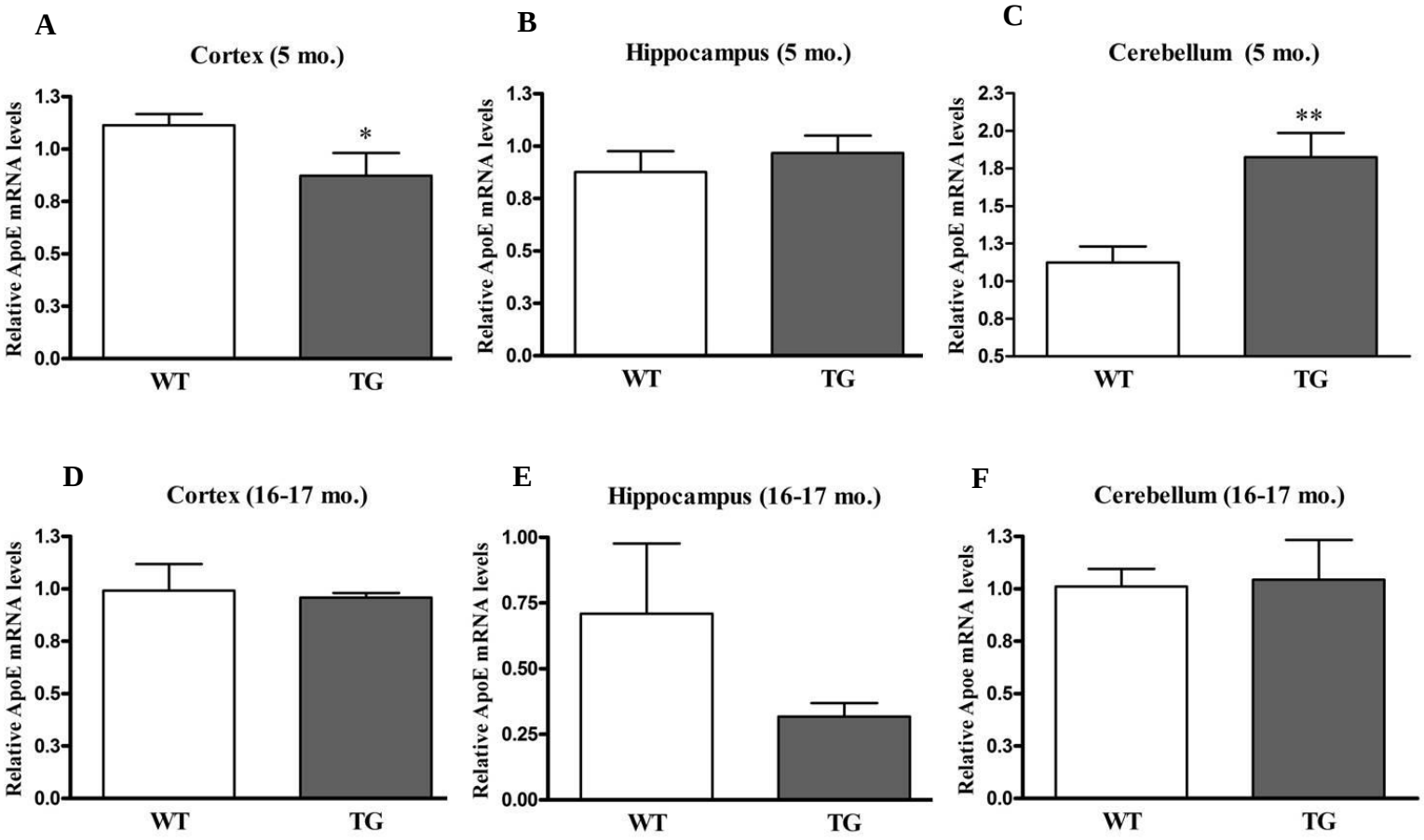

Fig. 29 Different microglial ApoE expression in 5 and 16-17 months old rats. Quantitative real-time PCR analysis revealed significantly lower ApoE mRNA levels (A) in the cortical and (C) in the cerebellar microglia of 5 months old rats. No differences in the group of older rats (D-F). Microglial ApoE mRNA expression was normalized to the housekeeping gene β-actin. Error bars represent mean ± SE. *$P < 0.05$; **$P< 0.01$; Student's t-test

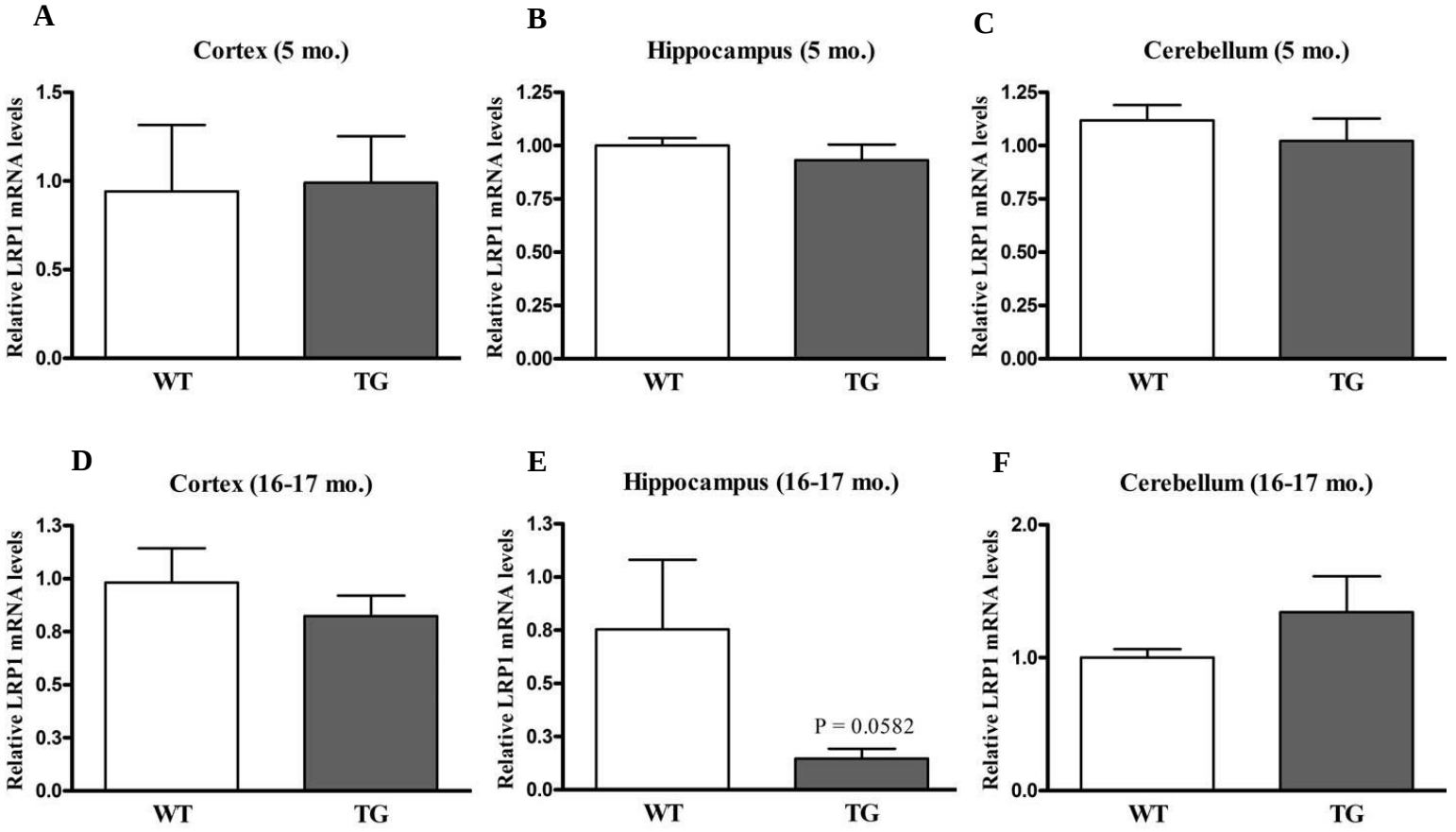

Fig. 30 Different microglial LRP1 expression in 5 and 16-17 months old rats. Quantitative real-time PCR analysis revealed almost significantly lower LRP1 mRNA levels (P=0.0582) in the hippocampal microglia of 16-17 months old rats (**E**). No differences in the group of younger rats (**A-C**). Microglial ApoE mRNA expression was normalized to the housekeeping gene β-actin. Error bars represent mean ± SE. Student's t-test.

CHAPTER 5:

DISCUSSION

Aim of the work presented in this thesis is to give new insights on the neuroprotection mediated by microglial cells and their conditioned medium, thus supporting the beneficial role of microglia, able to functionally sustain neurons in both physiological al pathological conditions.

First of all we started to characterize neuroprotective molecules produced and released by microglia, following their identification through proteomic analysis of media conditioned by rat primary microglial cultures. In recent years, several studies have been focused on strategies of immunomodulation to specifically increase neuroprotective phenotypes of microglial cells (Lockhart et al., 1994; Gunasekar et al., 1995; Zhou et al., 2005; Reynolds et al., 2009), mainly through the release of neuroprotective molecules, whose identification and characterization could be very important for developing novel therapies (Polazzi and Monti, 2010).

In particular the work described in part I and II has been focused on the investigation of the role of the proteins SOD1 and ApoE in this neuroprotective context. Their neuroprotective role has been mainly evaluated using a neurotoxicity model, namely exposure to 6-OHDA of CGNs, known to be fully protected by MCM (Polazzi et al., 2009). CGNs, a characterized population of homogenous primary neurons, are a good model to study the cellular, biochemical and molecular mechanisms of neuronal survival/death and are among the most widely-used neuronal primary cultures (Contestabile, 2002).

Finally, in part III will be discussed the experiments concerning the study of microglial M1/M2 phenotype in a rat model of Alzheimer-like amyloid pathology.

5.1 Superoxide dismutase 1

While superoxide dismutase is usually released by cells in its SOD3 isoform (Martin-Romero et al., 2002; Fatokun et al., 2007), SOD1 is also released by several cell lines (Mondola et al., 1996; Mondola et al., 1998; Cimini et al., 2002; Mondola et al., 2003). Here we show that SOD1 is produced and released by microglia and is able to counteract 6-OHDA-induced degeneration in CGNs.

From our data, SOD1 is constitutively released by cultured microglia, as it accumulates in the medium in the absence of experimentally induced activation. In particular, SOD1 concentration in the medium conditioned for 48 h has been estimated to be 0.04-0.05 U/ml (corresponding approximately to 10 ng/ml) and all the SOD1 secreted seems to be enzymatically active. Stimulation for 24 h with known pro-inflammatory activators of microglia (LPS and IFN-γ) did not affect neither SOD1 production nor its release. This lack of effect on SOD1 agrees with previous data showing that pro-inflammatory cytokines stimulate microglial expression of SOD2 and SOD3, but not SOD1 (Zelko et al., 2002; Chen et al., 2009). Interestingly, we demonstrate here that ATP increases the constitutive release of SOD1 in the medium, in agreement with a general modulatory response of microglia to this nucleotide (Inoue, 2008; Gyoneva et al., 2009). Release of SOD1 has been demonstrated to be ATP dependent through a microvesicle pathway in neuronal cell lines (Mondola et al., 2003), and secretion of several proteins occurs through a lysosomal secretory pathway in microglial cells (Bianco et al., 2005; Takenouchi et al., 2009). Two evidences argue in favor of this non-canonical secretion of the enzyme as a result of microglial ATP exposure: the rapid increase in SOD1 localization in secretory vesicles and the parallel increase in SOD1 released in the medium.

In addition, by adding exogenous SOD1 to a non-conditioned medium (although at concentrations remarkably higher than those present in the conditioned medium), we reproduced the neuroprotective effect towards CGNs treated with 6-OHDA. This raises the obvious question of the actual contribution of the low SOD1 concentration present in the neuroprotective conditioned medium. A possible explanation resides in the fact that the low SOD1 concentration present in the conditioned medium could cooperate with other neuroprotective factors, such as the ones mentioned in the introduction (TGF-β2, Apolipoprotein E, and plasminogen), to protect neurons (Polazzi et al., 2009). Consequently, it is not surprising that when SOD1 is added to a non-conditioned medium as the unique neuroprotective molecule, higher concentrations are required. In addition, the SOD1 increase in the medium conditioned for 24 h by primary microglia transfected with

plasmids expressing human wild-type SOD1 does not show any improvement in neuroprotection compared to the 24-hour standard conditioned medium, thus confirming that the amount of protein normally released by microglia is enough to grant protection.
Once that proteomic studies have definitely disclosed the pattern of neuroprotective molecules released by microglia in the medium, it could be possible to design specific mixtures of molecules granting neuroprotection at individually low concentrations through an additive/synergic effect. In this regard, it is relevant to consider that exogenous SOD1, even at very high concentrations, is not able to protect CGNs from neurotoxicity caused by other neurotoxic agents besides 6-OHDA. Therefore, the identification of other microglia-released factors involved in neuroprotection will likely contribute to explaining why other types of neurotoxicity (a shift of CGNs to non-depolarizing conditions or excitotoxycity) are significantly counteracted by MCM, but not by exogenous SOD1. To further complicate this situation, it has to be considered that SOD1 may act on target cells through both catalytic activity and receptor interaction (Mondola et al., 1994; Secondo et al., 2008).

In the present study, we started to characterize the mechanism of neuroprotection mediated through SOD1. We found that SOD1-mediated neuroprotection depends on increased cellular calcium deriving from an external source. Furthermore, we obtained preliminary evidence that this mechanism does not depend on the protein catalytic activity but it could be attributed to a receptor-mediated mechanism, as previously documented in neuroblastoma cells (Mondola et al., 2004). The fact that SOD1 concentration-dependent increase in Ca^{2+} entry into CGNs correlates with a decrease in neuroprotection in the presence of the Ca^{2+} chelator EDTA in the medium, strongly suggests that SOD1 neuroprotection is related to a intracellular [Ca^{2+}] increase from an extracellular source. This observation could open the way to future steps aimed at the full characterization of the signaling transduction pathway(s) elicited by SOD1. Moreover, identification of the intracellular Ca^{2+} increase as a mechanism involved in SOD1-mediated neuroprotection could also explain the lack of a SOD1 neuroprotective effect against other types of neurotoxic stimuli for CGNs, i.e. the shift to non-depolarizing conditions and the excitotoxycity of glutamate, as shown here.
In fact, Ca^{2+} entry from the extracellular compartment is limited if the neuronal membrane is not depolarized. This is what occurs with the shift to non-depolarizing conditions, since in this situation both voltage-sensitive Ca^{2+} channels and NMDA receptors, the main ways of entrance for Ca^{2+} in these cells, are inactive (Nakanishi and Okazawa, 2006).

Concerning glutamate excitotoxycity, it could be supposed that SOD1 is not neuroprotective because this type of neurodegeneration is mainly due to an excessive increase of intracellular calcium levels, leading to a condition of calcium overload (Nicholls and Budd, 1998). This excessive Ca^{2+} overload may mask the neuroprotective effect of the SOD1-mediated neuroprotection occurring at lower physiological levels of Ca^{2+} concentrations.

In order to further demonstrate the neuroprotective contribution of SOD1 to MCM, we tried to perform an siRNA knockdown of its expression. Unfortunately we obtained only a moderate decrease in SOD1 protein level, not sufficient to reduce MCM neuroprotection. Therefore, we used SOD1 inhibitors interfering either directly with Cu^{2+} bound to the enzyme (Forman et al., 1980) or indirectly through the action on the Cu^{2+} chaperon protein (Ogra et al., 1999). Therefore, the experiments performed were oriented at the abrogation of the neuroprotective effect of SOD1, either exogenously added to a non-conditioned medium or endogenously present in the conditioned medium.
Noteworthy, by decreasing the Cu^{2+} loading on SOD1, these inhibitors interfere not only with its enzymatic activity but also affect SOD1 structure and dimerization (Ding and Dokholyan, 2008). Accordingly, the inhibitor-mediated reduction in neuroprotection, observed here for both exogenous SOD1 and MCM, could be due to changes in SOD1 structure and to the consequent alteration of SOD1 interactions with receptors (Secondo et al., 2008).

Relevant to the present model, in which toxicity was delivered to CGNs through the dopaminergic toxin 6-OHDA, are previous results demonstrating that SOD1 gene transfer protects cultured nigral dopaminergic neurons from the same toxin (Barkats et al., 2002). Experiments could thus be devised in which microglia are forced to overproduce SOD1 to protect nigro-striatal dopaminergic neurons in models of experimental Parkinson-like neurodegeneration.

Mutations of SOD1 are found in the majority of familial cases of ALS and it has been demonstrated that protein mutations in microglia/macrophage cells are directly implicated in the disease (Beers et al., 2006; Boillee et al., 2006; Boillee and Cleveland, 2008; Henkel et al., 2009). Our novel evidence that normal SOD1 is constitutively released by non-inflammatory microglia and protects neurons may be relevant to better understanding the pathogenic/protective role of this protein, as well as of its release by microglia in ALS. A recent study showed that, in SOD1-related ALS mice, repopulation of the brain with microglia not expressing the pathogenic form of mutated SOD1 slowed

down motor neuron degeneration and prolonged lifespan (Beers et al., 2006). It is equally interesting that intraspinal infusion of wild-type SOD1 in a mouse model of ALS was able to reduce the damage in affected segments of the spinal cord, slow down disease progression, and prolong the lifespan of animals (Turner et al., 2005). These data strongly support the hypothesis that, in this neuropathology, there is not only a gain in microglial neurotoxic potential but also a loss of microglia physiological neuroprotective function (Appel et al., 2011). In this framework, our preliminary results obtained by transfecting primary microglia with plasmids expressing either human SOD1 wild-type or the pathogenic G93A and A4V mutations, linked to inherited ALS (Valentine et al., 2005), demonstrate that mutated SOD1 is released by microglia, but at lower levels compared to wild type. We can thus postulate that these mutations are able to inhibit the physiological secretion of SOD1 by microglia. Furthermore, the medium conditioned by microglia expressing mutated SOD1 (G93A) is not neurotoxic by itself, but its neuroprotective activity seems to be partially reduced, at least in our model of neurotoxicity. This is not surprising as it has already been suggested that SOD1 transgenic microglia have a reduced neuroprotective function (Sargsyan et al., 2011). However, further experiments must be performed to test these media on different types of neurotoxicity, and eventually in different neuronal cell types.

Note: some sentences have been cited verbatim from Polazzi, Mengoni et al., 2013.

5.2 Apolipoprotein E

Concerning Apolipoprotein E, it is well known that microglial cells are able to express it in physiological situation (Nakai et al., 1996). This has been confirmed by many studies both *in vitro* and *in vivo* (Saura et al., 2003; Mori et al., 2004; Qin et al., 2006), even though contrasting results have also been reported (Fujita et al., 1999; Xu et al., 2006).

The experiments showed in this thesis clearly demonstrate that rat primary microglial cells express ApoE. In addition it is constitutively released and accumulates in the medium (estimated: 130 pmol/ml), representing one of the most abundantly proteins secreted by microglia.

Microglia represent a "hybrid" brain cell, sharing characteristics of both neural and immune cells. Similarly to macrophages, microglia present complex protein secretion mechanisms, including the classical constitutive ER-Golgi pathway and regulated secretion, the latter including secretory granules and the lysosomal secretory pathway (Kockx et al., 2008). For instance, microglia use the lysosome secretory pathway for the release of interleukin-1β and SOD-1 (as described in paragraph 5.1), through the activation of the purinergic P2X7 receptor by ATP (Liu et al., 2008; Takenouchi et al., 2009; Polazzi et al., 2013). Moreover, similarly to other immune cells, microglia are able to externalize proteins, mRNA and miRNA through exosomes and microvesicles, which can be taken up by target cells (Thery et al., 2009). For the first time, we show here that microglia release ApoE through the Golgi apparatus, as happens in macrophages (Kockx et al., 2008). Although constitutive secretion and regulated exocytosis have been traditionally considered quite distinct, in macrophages ApoE appears to combine constitutive secretion with regulated exocytosis (Kockx et al., 2008; Karunakaran et al., 2013). The same type of ApoE secretion was also observed in rat astrocytes (Boyles et al., 1985).

Considering the involvement of ApoE in human neurological disorders, it is important to investigate what happens to ApoE release also in case of microglia activation, a common feature of most neurodegenerative diseases (Khandelwal et al., 2011; Czirr and Wyss-Coray, 2012), which can considerably modify microglial protein release (Takenouchi et al., 2009). As we previously did for SOD1, we decided to analyze the effect of microglial activation on ApoE release by using two activating stimuli, i.e. LPS and ATP. Both stimuli decreased ApoE microglial secretion, the effect of LPS being in agreement with previous data (Saura et al., 2003; Mori et al., 2004). We show here, as a novel observation, that microglia stimulation through the ATP/P_2X_7 receptor had the same effect of LPS on ApoE release. Interestingly, while LPS stimulation is considered to mimic

inflammation, ATP has been defined as a "warning molecule", being involved in both physiological and pathological conditions, thus representing a less aggressive signal in the neuron-glia cross-talk (Inoue, 2008; Butt, 2011). Taken together, our present results suggest that any alteration leading to increased microglial activation results in down-regulation of ApoE release. These data seem to be in contrast with *in vivo* observations, where microglial activation directly elicited by LPS infusion or induced by brain injury results in increased ApoE levels (Bales et al., 2000; White et al., 2001; Domenger et al., 2012). Based on previous results on down-regulation of microglial inflammatory state by ApoE (Laskowitz et al., 2001; Lynch et al., 2001; Guo et al., 2004), it is possible to postulate that *in vivo* increased ApoE production from activated microglia is part of an auto-regulatory feedback mechanism, which controls neuroinflammation (Barger and Harmon, 1997; Laskowitz et al., 2001).

These data, however, were obtained using a pure-microglia *in vitro* system, lacking other brain cells, mainly neurons, able to physiologically modulate microglial activation as well as diffusible factors secretion (Inoue, 2008; Jurgens and Johnson, 2012; Codolo et al., 2013). Whether and how neurons modulate microglial ApoE release remains still unknown. In order to fill this gap, we used neuronal conditioned media and neuron-microglia co-cultures (Polazzi et al., 2001; Polazzi et al., 2009). Although these models are definitely much simpler than the *in vivo* situation, they mimic the actual neuron-microglia interactions with good reproducibility (Facci and Skaper, 2012). To this purpose, we used two alternative approaches: i) exposure of microglial cells to medium previously conditioned by mature CGNs for 24 h and ii) co-culture experiments, in which microglial cells were directly plated over mature CGNs. From these experiments, we demonstrated for the first time that diffusible signals from healthy neurons influence microglia to increase their secretion of ApoE. A further important observation is that ApoE release induced by CGNm is differently modulated by microglial activators. In fact, while the stimulation with LPS strongly down-regulates neuronal-induced secretion of ApoE, the ATP-mediated activation through P_2X_7 receptor does not.

Moreover, in co-culture experiments we demonstrated that neurons when challenged with 6-OHDA induce lower microglial ApoE release levels compared to healthy neurons, even though microglia is significantly neuroprotective in co-cultures under 6-OHDA stimulation. This is in contrast with *in vitro* results obtained in mixed neuron/glia co-cultures, demonstrating an induced ApoE as a consequence of neuronal injury (Petegnief et al., 2001). In that study, however, the presence of astrocytes, which

secrete ApoE and induce microglial ApoE secretion (Mori et al., 2004), could explain the difference with our results obtained in astrocyte absence. An additional possible reason for reduced ApoE secretion in our neurodegenerating conditions, could be that ApoE is less concentrated in the medium of microglia-CGNs co-cultures due to neuronal uptake (Beffert et al., 1998; Williams et al., 1998). Several published data support this hypothesis: in *in vitro* systems, injured neurons internalized ApoE secreted by astrocytes (Yin et al., 2012) and neurotoxic β-amyloid increased ApoE internalization in cultured neurons (Beffert et al., 1998; Williams et al., 1998). Furthermore, in an *in vivo* model of brain injury, glial cells increased expression and release of ApoE. This happens with a concomitant increase of intracellular ApoE in degenerating neurons, likely due to internalization of released protein (Grootendorst et al., 2000; Xu et al., 2006). Another explanation could be that in our co-culture experiments physical interactions between neurodegenerating neurons and activated microglia, induced the latter, to strongly reduce ApoE secretion. This last hypothesis is also supported by our immunofluorescence result. In fact, 6-OHDA treatment in microglia-CGN co-cultures determined a change of ApoE staining in microglia: the protein dispersion and loss of Golgi ApoE localization similarly to what observed after BFA treatment, which leads to decreased microglial ApoE release. Therefore, it is likely that also in this case, the different ApoE localization in microglia is linked to the decreased ApoE release.

As mentioned before, ApoE shows neuroprotective properties in cultured neurons in several neurotoxicity models (Miyata and Smith, 1996; Drouet et al., 2001; Aono et al., 2002; Fuentealba et al., 2009). Here, we have demonstrated ApoE neuroprotection towards CGNs exposed to the toxin 6-OHDA, known to be fully protected by MCM (Polazzi et al., 2009; Polazzi et al., 2013). Moreover, silencing experiments demonstrated that ApoE is a necessary element for microglial neuroprotection of neurons challenged with 6-OHDA in co-culture conditions. Furthermore, the observation that ApoE, added at high concentrations to chemically defined not conditioned medium, confers neuroprotective properties, further supports an essential role of this molecule in neuroprotection. This result is in agreement with results from Qin and collaborators (2006), showing the essential role of microglial ApoE production in rescuing neurons from beta-amyloid toxicity in mixed neuronal cultures (Qin et al., 2006).

5.3 Microglial M1/M2 phenotype

Until now the majority of studies published on microglia have been performed using immortalized microglia-like cells and primary microglia derived from pre- or postnatal rat or mouse brain preparations (Gaikwad and Heneka, 2013). These cells, however, may only partly reflect the biology of microglial cells as they may lack substantial information which should only be acquired over time, once brain development has been finalized and the morphology and function of microglia has been shaped by neurotransmitters, endocrine factors, and their specific neuronal and astroglial environment. Based on this, one can assume that the experimental results obtained from *in vitro* studies using immortalized microglia like cells or primary microglia should be further backed up by the analysis of adult microglia (Gaikwad and Heneka, 2013).

Since not all of these studies can be done in the living animal, protocols are required which allow for the analysis of adult microglia in the context of a specific physiological state or disease. Microglial activation in response to physiological or pathophysiological stimuli will determine their morphology and function. Thus microglia can be polarized into various directions, of which two phenotypes have only recently begun to be investigated and described: the M1, usually associated to expression of pro-inflammatory cytokines, and the M2 phenotype, characterized by the expression of anti-inflammatory cytokines and promotion of tissue repair. It is likely that, with an increasing knowledge about microglia functions in the brain, further activation states will be discovered (Gaikwad and Heneka, 2013).

Considering all these observations we tried to characterize, here for the first time in three different areas (cortex, hippocampus and cerebellum), the microglial phenotype in the Alzheimer's pathology context, using the transgenic rat McGill-R-Thy1-APP.
In particular we analysed the mRNA levels of some important markers, known to characterize the M1 or the M2 microglial phenotype. Moreover we didn't culture the cells prior to mRNA extraction, in order to avoid alterations potentially ascribed to the culturing step. We thus obtained a "snapshot" of the mRNA expression in microglial cells harvested from 5 or 16-17 months old rats.

From the results obtained it is immediately evident that there are more variations between wild type and transgenic microglia in the pre-plaque stage (i.e. 5 months of age) compared to the microglia harvested from older rats. This may be surprising at first glance but might be explained by microglial senescence, that alters microglial function and ability to respond correctly to stimuli, a key factor contributing to progressive neurodegeneration,

according to recent evidence (Flanary et al., 2007; Streit et al., 2008; Luo et al., 2010). Moreover, this result is particularly promising if we consider the final aim of the study, i.e. find differences between wild type and transgenic microglia that may be linked to the pathology. The fact that we observed more variations in a phase that mimics the early stages of the pathology, can be particularly relevant for the identification of pharmacological therapies aimed at interfering with the development of the disease.

Surprisingly we observed also some variations in the cerebellum, initially considered as an internal control as a consequence of previous data published by the group, demonstrating that the mutant hAPP transgene expression provided by the *Thy*1.2 promoter was undetectable in lysates from cerebellum (Leon et al., 2010).

In addition, the cerebellum was traditionally considered a structure controlling motor regulation. However, in recent years it has been acknowledged that it exerts non-motor functions as well, such as cognitive, behavioral, and affective processing. Impairment of the cerebellum has been associated with cognitive functions since the recognition of the cerebellar cognitive affective syndrome (CCAS). This syndrome includes impairments in executive, visual-spatial, and linguistic abilities, with affective disturbance ranging from emotional blunting and depression to disinhibition and psychotic features, a set of symptoms that bears a striking similarity to many of the known symptoms of AD (Chen et al., 2010; Andersen et al., 2012). Importantly, neuronal loss in the cerebellum is part of the normal aging process in humans and the degeneration of Purkinje and granule cells has been reported in both familial and sporadic AD (Fukutani et al., 1996; Wegiel et al., 1999). Studies have shown diffuse amyloid plaques in the cerebellar cortex of AD patients and ubiquitin-immunoreactive dystrophic neurites, increased microglial density and evidence of astrocytosis are also found in the AD cerebellum (Dickson et al., 1990; Mattiace et al., 1990; Fukutani et al., 1996). However, despite these observations, the cerebellum is still widely regarded as being spared by Alzheimer's disease, often serving as a control tissue or a reference region in imaging studies (Chen et al., 2010).

Nevertheless, the differences in microglial M1/M2 markers expression between wild type and transgenic groups observed in our analysis highlight the importance of considering cerebellar neuropathological changes in the study of Alzheimer’s pathology. Moreover, in the older rats we found an increased expression of the astroglial marker GFAP in the transgenic samples compared to controls (data not shown). This could be due to an increased number of astrocytes in the original tissue, that may have compromised the

purity of the microglial preparation, thus confirming a possible cerebellar astrocytosis, frequent hallmark in the AD cerebellum, as mentioned before.

Considering the characterization of microglial M1 or M2 phenotype, from our results it is very difficult to reach a precise conclusion. Probably, as it has been suggested, the distinction in neurotoxic (M1) and neuroprotective (M2) microglia is an oversimplification and represents the extreme states reflecting the general attitude of microglia concerning their promotion of tissue injury or alternatively of tissue repair (Goldmann and Prinz, 2013).

Starting from the group of 5 months of age, we observed that lower levels of M2 markers were expressed in the cortex of the transgenic rats, thus suggesting that the anti-inflammatory and protective action of microglia might be compromised in the rats affected by the pathology. On the contrary, the situation in the hippocampus, particularly compromised by the pathology at this stage, was less predictable.

In fact, all the M1 and M2 markers (included TREM2, marker of phagocytosis) that showed a significant change between wild type and transgenic microglia were up-regulated in the hippocampus of transgenic animals. Interestingly, the same anti-inflammatory markers that are up-regulated in the hippocampus, are down-regulated or unchanged in the transgenic cortex or cerebellum, thus indicating a different microglial reactivity to the changes of the surrounding environment in these different areas.

Notably, microglia are present in large numbers in all major divisions of the brain but are not uniformly distributed. Considering the three areas here analyzed , hippocampus and cerebellum are respectively the most and the less densely populated, whereas cerebral cortex has an intermediate cell density in the normal adult mouse brain, a further evidence that these cells are sensitive to their microenvironment (Lawson et al., 1990).

Considering the group of older rats, significant differences were observed in the areas of cortex and hippocampus. In the hippocampus both pro- and anti-inflammatory markers resulted down regulated in the transgenic compared to wild type microglia. Therefore, the general trend observed in the hippocampus is opposite compared to the results obtained from the hippocampal samples of the younger rats.

In the cortex, only the M1 marker iNOS resulted downregulated in the transgenic microglia compared to wild type, a situation similar to the one observed in the hippocampal area.

Results suggest that, if at an initial step of the pathology these pro- and anti-inflammatory markers are differently expressed by microglia depending on the specific cerebral area considered, these differences tend to be reduced at an advanced step of the disease.

A possible explanation may be related to the fact that microglia senescence renders their activation response less reactive to the environment with aging (Streit et al., 2008).

Finally, we observed the microglial mRNA expression levels of the Apolipoprotein E and of one of its receptor LRP1, that interacts with the neuronally expressed amyloid precursor protein (APP) and regulates its proteolytical processing and the production of the Aβ peptide, a process that is of central importance for the pathogenesis of Alzheimer's disease (May et al., 2004). In the group of young rats the microglial ApoE mRNA levels in transgenic animals are down regulated in the cortex, unchanged in the hippocampus and up-regulated in the cerebellum, always compared to wild type. Considering the group of older rats, we couldn't observe any significant differences, except for a trend of ApoE mRNA down-regulation in the hippocampus. Interestingly this goes in parallel with another trend observed in the LRP1 analysis. Even though significant differences were not observed neither at 5 months nor at 16-17 months, a trend of LRP1 mRNA down-regulation was observed in the hippocampus of the group of older rats. This data could confirm the important role of ApoE, but also of LRP1 in the development of the pathology.

This study has provided an important contribution to the characterization of the McGill-R-Thy1-APP transgenic rat and is overall important for the characterization of the microglial role in the Alzheimer's pathology.

CHAPTER 6:

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