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Neuroinflammation and the role of glia: relevance for neurodegenerative and neurosupportive roles

Presentata da: **Elisa Motori**

Coordinatore Dottorato

Chiar. Prof.
Giorgio Lenaz

Relatore

Chiar.ma Prof.ssa
Silvana Hrelia

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ABSTRACT

Inflammation is thought to contribute to the pathogenesis of neurodegenerative diseases. Among the resident population of cells in the brain, astroglia have been suggested to actively participate in the induction and regulation of neuroinflammation by controlling the secretion of local mediators. However, the initial cellular mechanisms by which astrocytes react to pro-inflammatory molecules are still unclear. Our study identified mitochondria as highly sensitive organelles that rapidly respond to inflammatory stimuli. Time-lapse video microscopy revealed that mitochondrial morphology, dynamics and motility are drastically altered upon inflammation, resulting in perinuclear clustering of mitochondria. These mitochondrial rearrangements are accompanied by an increased formation of reactive oxygen species and a recruitment of autophagic vacuoles. 24 to 48 hours after the acute inflammatory stimulus, however, the mitochondrial network is re-established. Strikingly, the recovery of a tubular mitochondrial network is abolished in astrocytes with a defective autophagic response, indicating that activation of autophagy is required to restore mitochondrial dynamics. By employing co-cultivation assays we observed that primary cortical neurons undergo degeneration in the presence of inflamed astrocytes. However, this effect was not observed when the primary neurons were grown in conditioned medium derived from inflamed astrocytes, suggesting that a direct contact between astrocytes and neurons mediates neuronal dysfunction upon inflammation. Our results suggest that astrocytes react to inflammatory stimuli by transiently rearranging their mitochondria, a process that involves the autophagic machinery.

È ormai assodato che la neuro infiammazione costituisce una caratteristica comune a numerose patologie neurodegenerative. Tra le cellule gliali che mediano la risposta infiammatoria nel sistema nervoso centrale, gli astrociti rivestono un ruolo particolarmente importante in quanto, oltre a rispondere allo stimolo di molecole pro-infiammatorie esogene o prodotte dalla stessa microglia attivata,

producono anche fattori di crescita e neurotrofine essenziali per la sopravvivenza della cellula neuronale. Tuttavia, i meccanismi subcellulari che mediano la prima risposta degli astrociti a stimoli pro-infiammatori non sono ben noti. I nostri dati suggeriscono che i mitocondri sono uno dei primi target a rispondere all'infiammazione. Come dimostrato da indagini di microscopia time-lapse, l'infiammazione induce alterazioni delle dinamiche e della morfologia mitocondriali, con formazione di cluster mitocondriali perinucleari. Questi fenomeni sono accompagnati da un incremento nella produzione di ROS e dall'induzione di autofagia. Tuttavia le alterazioni sopra descritte sono transienti, dal momento esse si risolvono nell'arco di 24-48h. In particolare, la rimozione dell'autofagia determina l'impossibilità da parte degli astrociti di ripristinare una corretta funzionalità mitocondriale in seguito ad infiammazione, indice del fatto che l'autofagia svolge un ruolo-chiave nel quality control dei mitocondri in questo modello. Abbiamo inoltre investigato gli effetti mediati dagli astrociti sulla vitalità neuronale in un sistema di co-culture, e abbiamo osservato che l'infiammazione induce neurotossicità: lo stesso effetto viene a mancare se si effettua l'aggiunta di mezzo condizionato da astrociti infiammati su colture pure di neuroni, suggerendo che nel nostro modello il contatto diretto tra astrociti e neuroni è fondamentale per indurre disfunzione neuronale a seguito di infiammazione. Nell'insieme, questi dati sugggeriscono che l'infiammazione induce alterazioni temporanee della funzionalità mitocondriali associate ad autofagia.

INTRODUCTION

1 Specific role of astrocytes in neuron-glia communication during neuroinflammation

Glial cells represent the most prominent population of cells residing in the brain. They were first discovered by Rudolf Virchow in 1846, who named this abundant population *nervenkitten* ("glue", from the Greek name *glia*), but it was only at the beginning of the 20th century that scientists started to identify in glial cells an heterogenous population, particularly thanks to the contribution of Ramon y Cajal and Rio Hortega, who identified microglia and oligodendrocytes by using metallic impregnation of tissue samples (Wang and Bordey 2008). Starting from then, microglia and oligodendrocytes have progressively acquired more importance among the cells of the central nervous system (CNS), mostly due to their highly specialized roles in exerting immune functions (microglia) and in myelinating axons of projecting neurons (oligodendrocytes).

Importantly, a third type of glial cells, originally defined macroglia but known with the name of astrocytes, constitute the most abundant fraction of cells with a glial phenotype in the brain (80% of all glia), yet this population of cells has long been neglected by scientists. Always described as the cement of the brain and characterized by "passive" functions such as to provide a scaffold for the proper positioning of developing neurons, astrocytes (and their counterpart in the peripheral nervous system, the Schwann cells) gained significant attention only during the last 20 years, when it was first discovered that these cells do express voltage-gated channels on their membrane (Bevan, Chiu et al. 1985). With time, it became clearer that astrocytes actively cover important roles in neuronal functioning and homeostasis: they are strategically positioned between neurons and blood vessels, with their fine processes engulfing the neuronal synapses at one side and intimately interacting with the walls of blood vessels at the other (astrocytic end-feet), in a

manner that morphologically reflects the unique capacity of these cells to directly coordinate neuronal activity with the local signals released by non-neuronal cells. As a consequence, it is not entirely surprising that astrocytes have been soon suggested to play important roles in the pathogenesis of a variety of CNS disorders (Barres 2008), including widespread neurodegenerative diseases such as the Alzheimer's and Parkinson's diseases (Sofroniew and Vinters 2010). In this evolving scenario, the question arises what is the precise contribution of glial cells, in particular of astrocytes, to the initial phases of neurodegeneration or, rather, if astrocyes possess a neuroprotective potential in this respect. Characterizing astrocytic reactivity and behavior before, during and after the establishment of these diseases could open the avenue to the discovery of new therapeutic approaches aimed to prevent - or restore - neuronal viability and circuits function.

1.1 Astrocytes: the Kali of the brain

Talking about astrocytes, the first important issue - yet partially unsolved - is what exactly defines a cell as "astrocyte". In fact, for long time astrocytes have been viewed as a homogenous population of cells characterized by a star-shaped morphology contacting both neurons and blood vessels. The advent of mouse genetics and of new tools based to the selective expression of fluorescent reporter molecules driven by astrocytic-specific promoters (for instance the Glial Fibrillary Acidic Protein, GFAP) (ref) (Nolte, Matyash et al. 2001) have dramatically contributed to redefine the identity of astrocytes, in particular by revealing their heterogeneity in morphology, antigenic phenotype, location and function.

Astrocytes can be classified in at least two different classes, depending on their morphology and location, which also reflect differences in functions (Volterra and Meldolesi 2005). The vast majority of astrocytes fall into the "protoplasmic" phenotype (or type I), which is characterized by irregular morphology and very dense branched processes: these astrocytes generally express GFAP and principally populate the grey matter. A distinctive feature of these astrocytes is that their processes ensheat synapses and neuronal cell bodies but they also possess end-feet terminals which embrace blood vessels (Volterra and Meldolesi 2005). On the other

side, fibrillary (or fibrous or type II) astrocytes are commonly less immunoreactive for GFAP, although they express the calcium binding protein S100β (as protoplasmic astrocytes also do), and show a simpler morphology with thinner processes in comparison with type I astrocytes. Type II astrocytes are much frequently observed in the white matter, where they contact nodes of Ranvier. Curiously, protoplasmic and fibrillary astrocytes also differ in their electrical properties (Table1) and for the presence/absence of gap-junctions.

	Protoplasmic	**Fibrillary**
Input resistance	low	high
Membrane potential	very negative	negative
Potassium currents	voltage and time-independent	voltage-dependent
Glutamate uptake	different	low

Table 1. Different electrical properties of astrocytes.

Besides these two main categories, dedicated types of astrocytes are present in the retina (Müller glia) and cerebellum (Bergmann glia), regions in which they support and modulate the functions of specialized local neurons (Hirrlinger, Hulsmann et al. 2004). A last specialized type of astrocytes is present in those restricted regions of the adult brain in which neurogenesis persists: the subependimal zone of the lateral ventricles and the dentate gyrus of the hippocampal formation (Suh, Deng et al. 2009). In all these regions, astrocytes have been classically identified by using morphology and marker expression profile. In particular, a widely used marker for identifying and labeling astrocytes is the GFAP, which is very strongly expressed in layer I of the cerebral cortex, in the hippocampus, in the neurogenic areas and in the cerebellum (Fig. 1). Yet, by the moment these mature, differentiated astrocytes (like those located in the cortex) exit from their usual resting state, such as following injury (Sofroniew 2009), inflammation (Farina, Aloisi et al. 2007) or the insurgence of neurodegenerative diseases (Thal, Hartig et al. 1999),

GFAP becomes up-regulated and is thus also commonly used as a marker to identify the so called reactive state of astrocytes (or reactive gliosis). For this reason, primary cultures of astrocytes are typically very immunoreactive for GFAP, since after their dissociation and growth in culture these cells frequently divide and maintain an undifferentiated state compared to the in vivo situation.

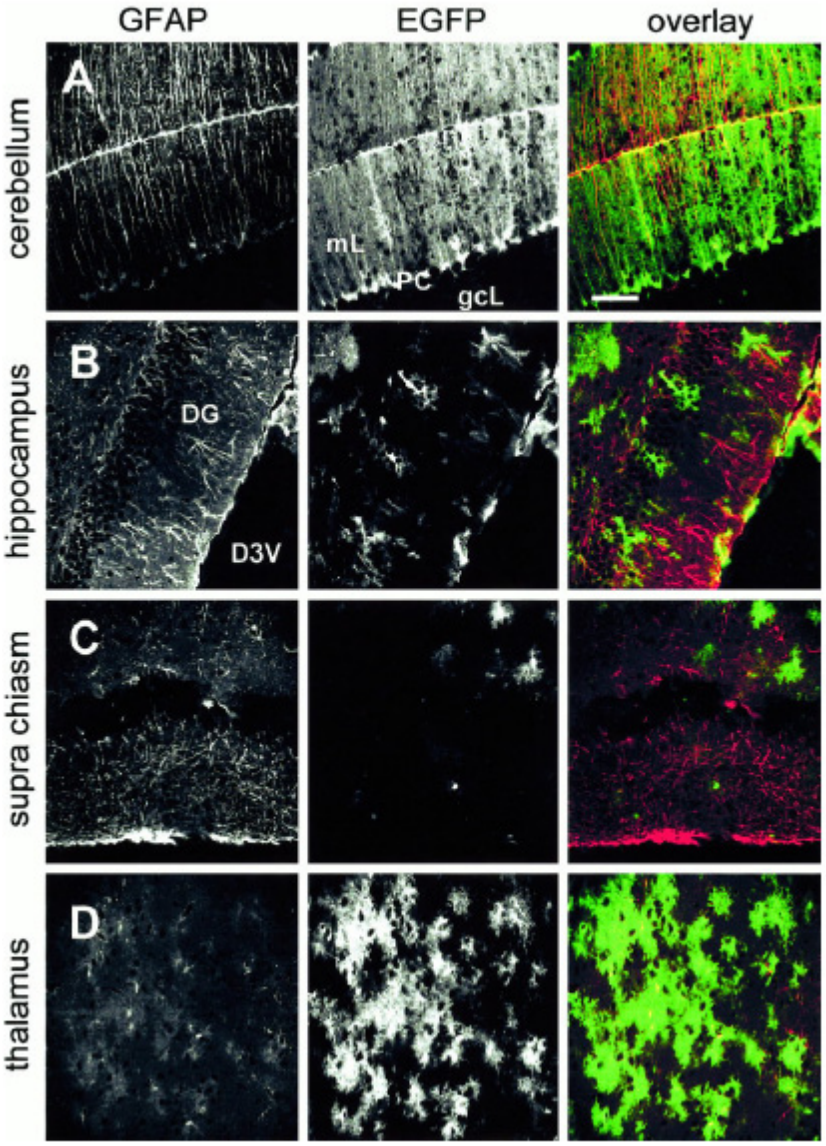


Figure 1. **Heterogeneity of GFAP positive cells with respect to their morphology and location.** Studies using transgenic mice expressing fluorescent protein driven by the GFAP promoter revealed

morphological and topological differences in the population of astroglial cells. This picture, taken from (Nolte, Matyash et al. 2001), shows the extreme variability of GFAP positive cells.

In addition to GFAP, astrocytes in the CNS express a series of markers which helps for their identification: S100β (calcium-binding protein) mostly labels the astrocytic cytoplasm, whereas Glt-1 and GLAST (glutamate transporters) are expressed in the membrane of astrocytes. However, caution should be used when using these markers for classifying astrocytes. In fact, S100β immunoreactivity not only labels a subtype of mature astrocytes, but it could be observed in Neurogenin 2 (NG2) expressing cells, which are commonly referred as oligodendrocytes precursors (Nishiyama, Chang et al. 1999). Similarly, the glutamate transporter Glt-1 is not exclusive for astrocytes, but can also label oligodendrocytes (D'Amelio, Eng et al. 1990). More recently, gene profile analysis has identified new astrocyte-specific genes. Amongst them, Aldh1L1 (aldehyde dehydrogenase 1 family, member L1) possesses a wide range of expression in astrocytes in comparison to other markers (Cahoy, Emery et al. 2008).

1.2 Astrocytes physiology

Typically, type I astrocytes (the most abundant in the cerebral cortex) display a complex branching of their processes. Single-cell microinjection of a fluorescent dye that can diffuse across gap-junctions has revealed that individual astrocytes cover discrete territories with little or minimal overlap with adjacent astrocytes (Bushong, Martone et al. 2002) (Fig. 2). This architectural arrangement has been suggested to be theoretically ideal for maximizing the control exerted by astrocytes over the thousands of synapses that are located within these small territories, a function which appears to correlate with the high motility of astrocytic lamellipodia and filopodia located at the tips of their processes (Hirrlinger, Hulsmann et al. 2004; Nishida and Okabe 2007; Reichenbach, Derouiche et al. 2010; Lavialle, Aumann et al. 2011). Classical studies of electron microscopy, in which ultra-thin sections of tissue have been analyzed, revealed an intimate connection between these astrocytic

processes and single synapses (Bezzi, Gundersen et al. 2004; Halassa, Fellin et al. 2007) such that scientists have recently proposed the term “tripartite” synapse to include astrocytes into the fundamental structure (the synapse) at the core of synaptic transmission. During the last 10-15 years, numerous studies have clearly demonstrated that this structural proximity is functional to the role of astrocytes during synaptic transmission: these cells can indeed modulate, in various manners, the excitability of neurons and their cellular state (Bezzi, Gundersen et al. 2004; Halassa, Fellin et al. 2007; Hamilton and Attwell 2010). The importance of astrocytes in this sense is such that a complete set of molecules is now part of the so called class of “gliotransmitters”: as the name says, they are released by astrocytes and can modulate the excitability of other cells as the neurotransmitters released by neurons do (Volterra and Meldolesi 2005; Hamilton and Attwell 2010).

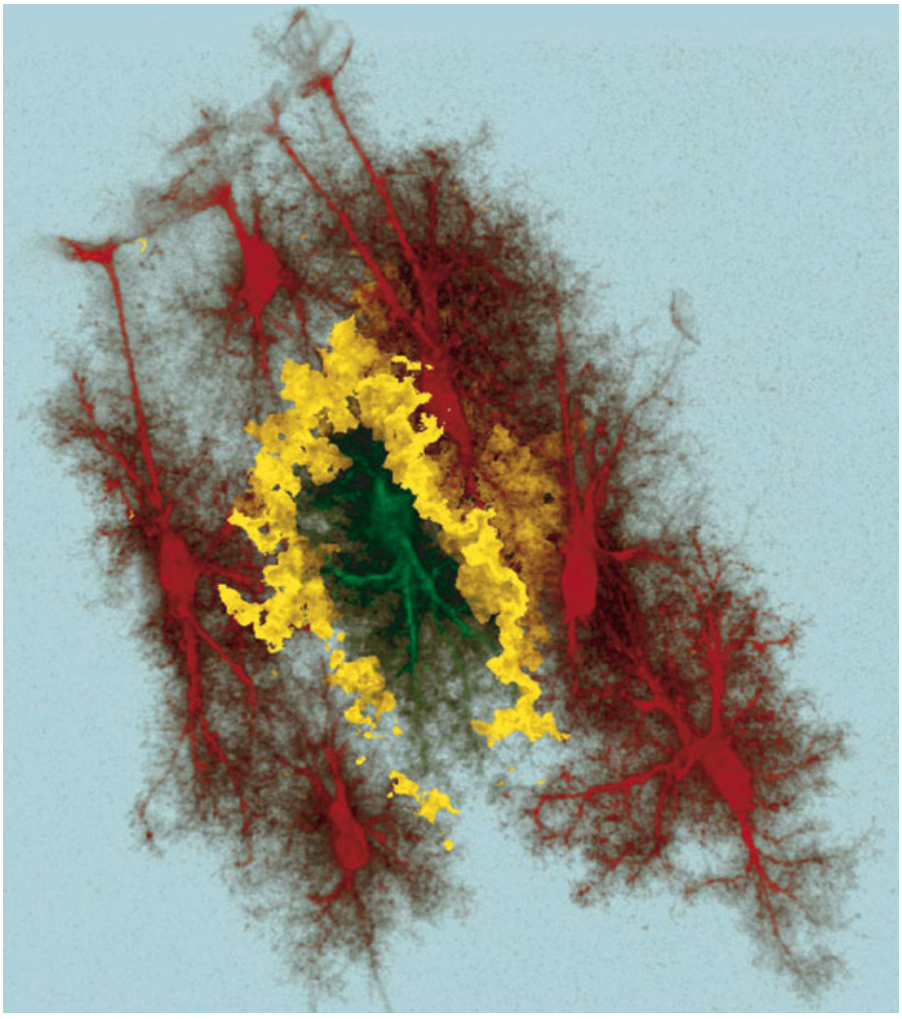


Figure 2. **3D view of astrocytes architecture.** Single-cell microinjections of different dyes reveals the discrete territories of astrocytes in vivo. Picture modified and taken from (Bushong, Martone et al. 2002)

Gliotransmitters comprise mediators such as ATP, glutamate, D-serine, trophic factors and others (Volterra and Meldolesi 2005; Perea, Navarrete et al. 2009). Interestingly, all these molecules can potentially impact the functional state of local synapses, and several demonstrations of such effects in astrocytes-neurons co-cultures and in slice tissues have been convincingly provided (Pasti, Zonta et al. 2001; Yang, Ge et al. 2003; Fellin, Pascual et al. 2004; Pascual, Casper et al. 2005; Perea and Araque 2007; Navarrete and Araque 2010; Santello, Bezzi et al. 2011; Navarrete, Perea et al. 2012). But how exactly the secretion of gliotransmitters by astrocytes is triggered, and what is their physiological role? A critical point regarding this issue consists in the mode through which perisynaptic astrocytes sense the ongoing neuronal activity and integrate this signal with those collected from the non-neuronal environment (extracellular space, blood vessels, ect.). Astrocytes are indeed equipped with a full set of membrane receptors which allow them to signal following synaptic release or spillover of classical neurotransmitters (glutamate or γ-amino butyrric acid, GABA), purinergic mediators (Volterra and Meldolesi 2005) and molecules associated with the inflammatory reaction (Glass, Saijo et al. 2010). Intriguingly, the common mode by which astrocytes integrate the signaling pathways initiated by these molecules is via modulation of local (at the level of single processes) and global (cell body) transients of calcium (Ca^{++}) (Araque, Carmignoto et al. 2001; Di Castro, Chuquet et al. 2011), a mechanism which is as well employed for astrocyte-astrocyte communication (astrocytic Ca^{++} waves) through gap-junctions (Carmignoto 2000; Araque, Carmignoto et al. 2001; Kuga, Sasaki et al. 2011). In turn, astrocytes respond to these transients by secreting gliotransmitters (in particular glutamate) into the local environment, and therefore could modulate, in this manner, the efficiency of synaptic transmission and synaptic homeostasis (Volterra and

Meldolesi 2005; Perea, Navarrete et al. 2009). These findings have led to the idea that neuron-glia intercommunication is much more dynamic than previously believed. In addition, there is now growing evidence that molecules linked to inflammatory reactions, such as the cytokine tumor necrosis factor-alpha (TNF-α) and prostaglandins (Bezzi, Carmignoto et al. 1998; Santello, Bezzi et al. 2011), which are likely to be secreted during neuroinflammation in many pathological conditions, could play a critical role in disrupting the normal cross-talk between neurons and astrocytes, thus contributing to the development of the diseases.

1.3 Mechanism of neuroinflammation

Inflammation is classically defined as a highly regulated biological process that, by means of both the innate and adaptative immune systems, enables the host organism to deal with, and finally eradicate, the infection (Allan and Rothwell 2003; Medzhitov 2008). Although somehow isolated from the systemic circulation by the blood-brain barrier, the brain is an organ which undergoes dynamic immune responses. It possesses a resident type of immune cells, the microglia, which under physiological conditions constantly monitor the local microenvironment and communicate with astrocytes and neurons by secreting anti-inflammatory molecules and neurotrophic factors (Glass, Saijo et al. 2010). Following injury or pathogen invasion, however, microglial cells become activated and promptly release a completely different set of factors, such as pro-inflammatory cytokines and chemokines, which contribute to the onset of the local inflammatory response (Gonzalez-Scarano and Baltuch 1999). At the same time, these microglial-released mediators engage immune cells from the peripheral system in order to facilitate tissue repair (Hickey and Kimura 1988; Glass, Saijo et al. 2010). Thus, inflammatory cytokines and chemokines released in the site undergoing inflammation mainly serve to halt the pathogen invasion. Inflammation presents features of a self-limiting response, and it is typically resolved once the cause of infection has been removed. Nevertheless, when the inflammatory stimuli are persistent or the usual mechanisms appointed to resolve inflammation become overwhelmed, the prolonged secretion of

pro-inflammatory molecules amplifies the production of neurotoxic species, with known detrimental consequences for tissue homeostasis (Rivest 2009).

A typical inflammatory response is initiated following the interaction of a pathogen with a specific class of membrane receptors, the so called pattern recognition receptors (PRR). So far, four different types of PRR have been identified: Toll-like receptors (TLRs), C-type lectin receptors, cytoplasmic proteins such as the RIG-1 like receptors and NOD-like receptors (Rivest 2009; Takeuchi and Akira 2010). Among them, TLRs are widely expressed in glial cells, in particular by microglia and astrocytes, whereas to a minor extent in neurons (Kielian 2006; Konat, Kielian et al. 2006). These receptors recognize a complex pattern of molecules specifically expressed by pathogens (pathogen-associated molecular patterns, PAMP). Between the types of TLRs that have been characterized both in humans and mice (up to twelve), of particular interest is TLR4 - which binds the lipopolysaccharide (LPS) component of the Gram-negative bacteria - and TLR3, which is specific for the binding of double-strand RNA (Hanke and Kielian 2011).

PRR can, in some cases, sense endogenous components as well, such as molecules released from necrotic cells and heat-shock proteins, thus acting as receptors for potential danger signals (Asea, Rehli et al. 2002). Several evidences suggest a critical role of TLRs, especially TLR2 and TLR4, in the etiology of chronic inflammatory diseases, such as atherosclerosis and type-2 diabetes (Balistreri, Colonna-Romano et al. 2009). Besides PRR, microglia and astrocytes express also other types of receptors which can equally prompt inflammation such as (i) purinergic receptors, that sense ATP released from cells upon cell death or injury (Di Virgilio, Ceruti et al. 2009), and (ii) scavenger receptors, which bind oxidized proteins and lipids (Murgas, Godoy et al. 2012).

The major downstream signaling pathways activated upon ligation with PRR are the I-kappa-B (IkB) and Mitogen-Activated Protein Kinases (MAPK). In turn, these kinases modulate several transcription factors belonging to the families of Nuclear Factor kB (NF-kB), the Activator Protein AP-1 and the Interferon Regulatory Factors IRFs, which further control a wide set of genes depending on the cell type (Smale 2010). Accordingly, many of these genes encode for pro-

inflammatory cytokines such as Tumor Necrosis Factor α (TNF-α), Interleukin-β (IL-1β) Interleukin-6 (IL-6), which contribute to the amplification of the inflammatory response, chemokines (whose role is to recruit additional immune cells) and antimicrobial proteins (e.g. iNOS) that orchestrate several cellular functions like cell motility and intracellular killing. Moreover, the increased production of reactive oxygen or nitrogen species (ROS or RNS) via the NADPH oxidase system, or by mitochondria, further enhances the antimicrobial response but can as well promote collateral damages to the cell.

In contrast to the above described mechanisms which contribute to the generation and amplification of inflammation, the nervous system has several counter-regulating mechanisms to control and terminate the inflammatory response. In general, these mechanisms involve the induction of transcriptional repressors (Nuclear Receptor Related-1, Nurr1), proteins that specifically inhibit signal transduction pathways (e.g. Suppressor of Cytokine Signaling, or SOCS, proteins) and anti-inflammatory mediators, such as Interleukin-10 (IL-10) and Transforming Growth Factor-β (TGF-β). Yet, how the nervous tissue finely controls the balance between pro-inflammatory molecular pathways and counter-regulating mechanisms represents a critical question currently unsolved.

1.4 Contribution of neuroinflammation to neuronal dysfunction and degeneration

It is now widely accepted that when the inflammatory response of the nervous tissue (neuroinflammation) is not properly controlled, it can significantly contribute to neurodegeneration. As a matter of fact, neuroinflammation accompanies the onset and the progression of widespread neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's diseases (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). All these syndromes are indeed characterized by a general activation of microglial cells (Gonzalez-Scarano and Baltuch 1999; Glass, Saijo et al. 2010). Massive activation of both microglia and astrocytes, in combination with elevated levels of cytokines, has been found to surround the senile plaques in brain samples of AD patients (Cartier, Hartley et al. 2005). Interestingly, traumatic injury

and infection has been suggested to contribute to the early stages of AD (Migliore and Coppede 2002), and there is now evidence for a correlation between chronic inflammatory diseases and a higher risk to further develop AD (Biessels, De Leeuw et al. 2006; Biessels, Staekenborg et al. 2006; van den Berg, Kessels et al. 2006; Granic, Dolga et al. 2009). Similarly, reactive gliosis has been reported in brain samples of PD patient (Damier, Hirsch et al. 1993; Miklossy, Doudet et al. 2006; Vroon, Drukarch et al. 2007; Whitton 2007). Despite the efforts made in order to elucidate the specific mechanisms underlying these diseases, their ethiopathogenesis is far to be understood. Although it is quite unlikely that neuroinflammation is the initiating factor of these diseases, a growing body of evidence supports the idea that chronic neuroinflammation not only underlies, but significantly participates to their progression. Importantly, studies indicate that the inflammatory response may take place even prior the loss of neurons in these diseases (Frank-Cannon, Alto et al. 2009), strengthening the necessity to understand the mechanisms regulating neuroinflammation and how they could contribute to neurodegeneration.

2 The autophagy-inflammation-mitochondria crosstalk

Autophagy is a well conserved biological process initially discovered as a mode of undergoing cell death opposed to apoptosis and necrosis (Debnath, Baehrecke et al. 2005; Levine and Yuan 2005). Its peculiarity is the formation of intracellular autophagosomes, specialized compartments which enter the degradation pathway through fusion with late endosomes and then lysosomes (He and Klionsky 2009). Despite the first observations and the classification of autophagy as a cell-death mechanism, during the last years it became clear that cells also use autophagy as a physiological process for recycling damaged molecules and organelles (Yang and Klionsky 2009). Different types of autophagy have been identified depending on which of the known intracellular signaling pathways finally leads to the formation of autophagosomes (Cuervo, Bergamini et al. 2005; He and Klionsky 2009; Klionsky, Codogno et al. 2010). Amongst them, mitophagy represents a specific subtype of autophagy which, as the name says, is important for removing unwanted

mitochondria (for example damaged ones) and at the same time allows for controlling the overall degree of mitochondrial health and number. Several lines of evidence support now the idea that impairments of the autophagic machinery in neurons critically contribute to neurodegeneration (Hara, Nakamura et al. 2006; Mizushima and Hara 2006). This assumption is based on the observations that inhibition of autophagy results in the accumulation of dysfunctional mitochondria (Hara, Nakamura et al. 2006), with an obvious detrimental impact on the bionergetic status of the cell, or in the impossibility to degrade disease-related proteins which could then accumulate, ultimately affecting cell viability (Cuervo, Bergamini et al. 2005; Massey, Kaushik et al. 2006).

Besides these classical roles of autophagy, recent reports indicate that this process possesses a wider spectrum of functions, some of which involve the regulation of diverse aspects of the innate and adaptative immunity (Mizushima, Levine et al. 2008; Stappenbeck, Rioux et al. 2011). For example, autophagy regulates the activation of the inflammasome (that is, the assembly of key proteins involved in inflammation into a multi-protein complex, for review see (Schroder and Tschopp 2010; Gross, Thomas et al. 2011)) either by direct degradation of the inflammasome complex (Harris, Hope et al. 2009) or, indirectly, controlling the generation of ROS at the level of single mitochondria (Saitoh, Fujita et al. 2008).

Taken together, these aspects suggest a complex scenario in which autophagy, inflammation and cell death are interconnected each other via multiple pathways: at the center of this interplay are mitochondria, which can be both targets and regulators of autophagy and inflammation. Given the well established involvement of dysfunctional mitochondria in the development of neurodegenerative diseases (Hara, Nakamura et al. 2006; Mizushima and Hara 2006; Mizushima, Levine et al. 2008), it becomes important to elucidate which mechanisms play a key role in mitochondrial dynamics and functioning and how these cope with the regulation of the inflammatory and autophagic pathways.

2.1 Autophagy

Autophagy is a self-eating cellular process highly conserved across species. This evolutionary conservation is likely due to the essential role played by autophagy in numerous physiological processes and stress conditions, such for instance during embryonic development or cytoprotection/survival following starvation (Jia and Levine 2007; He and Klionsky 2009), or when it's required to get rid of damaged organelles (Cuervo, Bergamini et al. 2005; Deretic and Klionsky 2008; He and Klionsky 2009; Amarnath, Flomerfelt et al. 2010). Depending on the mode through which the intracellular targets are finally delivered to lysosomes, autophagy can be classified in macroautophagy, microautophagy and chaperone-mediated autophagy.

Both macro and microautophagy are capable of either selective or non-selective mechanisms of degradation, distinguished depending on the target which becomes engulfed. Common selective processes are the ones which target mitochondria (mitophagy), peroxisomes (pexophagy), endoplasmic reticulum (reticulophagy) and ribosomes (ribophagy). Macroautophagy (hereafter named autophagy) occurs when cytoplasmic constituents, such as damaged organelles, molecules, or pathogens are sequestered into double-membrane structures defined autophagosomes. The fate of these autophagosomes is to fuse with lysosomes in order to promote the degradation of targeted cargoes (Bampton, Goemans et al. 2005; Massey, Kaushik et al. 2006; Klionsky, Elazar et al. 2008).

While the existence of autophagy was documented already in the 1950s, a time in which electron microscopy allowed the first observations of autophagosomal structures (Fedorko 1967), a more detailed description concerning the autophagic machinery and the autophagy related genes (Atg) was unraveled only much later, thanks to genetic screenings performed in yeast (Cao, Cheong et al. 2008; Cheong and Klionsky 2008; Cao, Nair et al. 2009). Further studies found homologues of those genes also in higher eukaryotic systems, suggesting that this process is conserved across species (Itakura and Mizushima 2010).

2.1.1 The autophagy core machinery

The autophagic process could be divided in four major steps (depicted in Fig. 3), each of them requiring a precise orchestration of a specific subset of Atg proteins for proper execution.

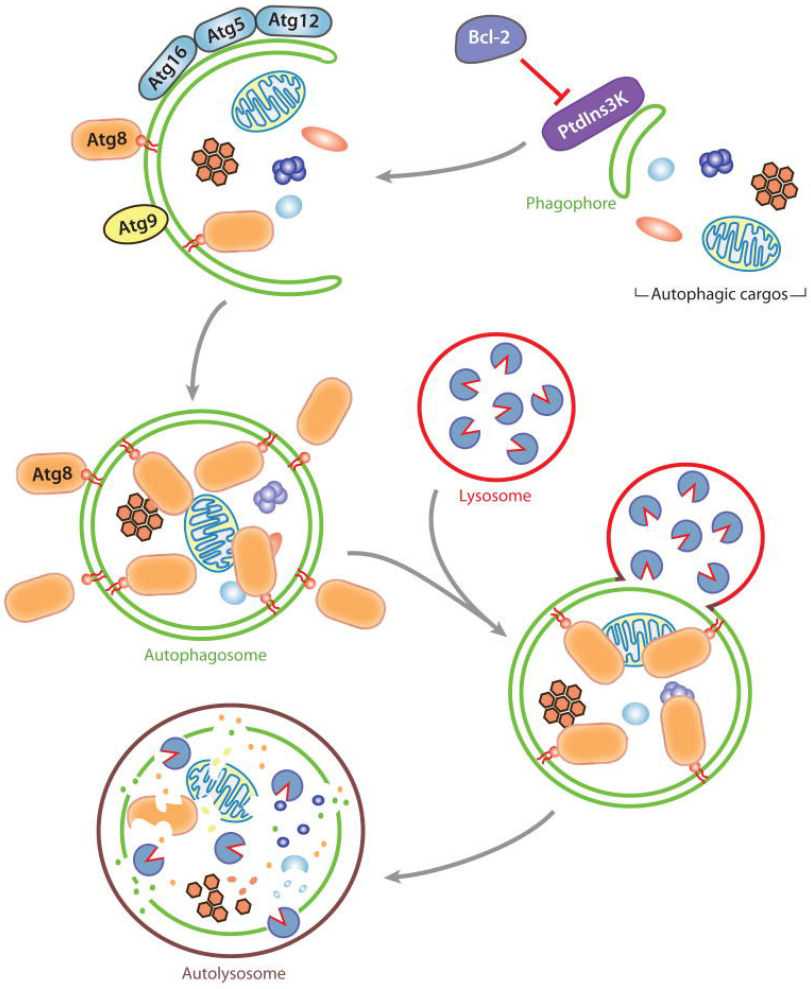


Figure 3. **Schematic view of autophagy**.
Picture taken from (He and Klionsky 2009).

The first step, the induction of autophagy, is critical because if not properly controlled, could be harmful for the cell. This step is maintained under control by the

serine/threonine kinase target of rapamycin (TOR), a protein that integrates signals coming from several intracellular pathways and, in turn, inhibits the Atg1 kinase (which mammalian homologues are ULK1 and ULK2) under physiological conditions (Cheong and Klionsky 2008; Cheong, Nair et al. 2008). When cells switch to starvation or the metabolic status becomes poor, then inhibition of TOR leads to the activation of Atg1 that, by recruiting other Atg proteins, forms a complex with the Focal Adhesion Kinase FIP200 (Hara and Mizushima 2009). This complex promotes the formation of the phagophore assembly site (PAS), constituting the initial compartment of the autophagosome, the phagophore (Cheong and Klionsky 2008).

In case of selective autophagy, an important step is the recognition of the cargo that can occur following binding of specific receptor proteins. Once this recognition is completed, Beclin-1 dissociates from the antiapoptotic protein Bcl-2 (Levine, Sinha et al. 2008) and binds to the class III phosphatidylinositol 3-kinase (PI3K) (Furuya, Yu et al. 2005; Criollo, Vicencio et al. 2007; Levine, Sinha et al. 2008; Itakura and Mizushima 2009). This last event then leads to the generation of the autophagosomal structure. The complex formed by Beclin-1 and PI3K is in fact essential for recruiting two interrelated ubiquitin-like conjugation systems, Atg12-Atg5-Atg16 and Atg8-phosphatidylethanolamine (Atg8-PE), to the phagophore site (Geng and Klionsky 2008; Xie, Nair et al. 2008; Xie, Nair et al. 2008; Itakura and Mizushima 2010). Both Atg12 and Atg8 must undergo an irreversible conjugation in order to be recruited: Atg12 is activated by Atg7 (ref); Atg8 (Light Chain 3B-I/LC3B-I in the mammals) is localized to the cytosol under nutrient-rich conditions, but upon induction of autophagy is first cleaved by a protease, Atg4, then transferred by the above mentioned Atg7 to Atg3 in order to be conjugated to PE. In this lipid-conjugated form, Atg8 (or LC3B-II) is incorporated within the membrane of the autophagosome.

The last steps of autophagy include the fusion of the autophagosome with a lysosome, a process mediated by the endosomal protein Rab7 (Romano, Gutierrez et al. 2007) and the lysosomal protein Lamp-2 (Romano, Gutierrez et al. 2007), and the

degradation of the internalized cargo by lysosomal proteases. The degraded products are then released back to the cytosol upon collapse of the structure.

2.1.2 Deregulation of autophagy in neurodegeneration

A growing body of evidence suggests the existence of a close link between an alteration in the autophagic machinery and the insurgence of several diseases such as cancer, infection, heart failure and neurodegeneration, as shown in Fig. 4 (Levine and Yuan 2005; Levine 2006; Mizushima, Levine et al. 2008). It has been previously mentioned that autophagy occurs at very low levels under physiological conditions, although differing depending on the given tissue. Yet, the basal levels of autophagy are kept under rigid control, since mice lacking important proteins of the autophagic machinery such Atg7, Atg5 or Beclin1, die during embryogenesis or immediately after birth (Kuma, Hatano et al. 2004; Komatsu, Waguri et al. 2005; Fimia, Stoykova et al. 2007), whereas conditional knockout mice survive, although exhibiting severe motor deficits and neuronal dysfunction (Hara, Nakamura et al. 2006).

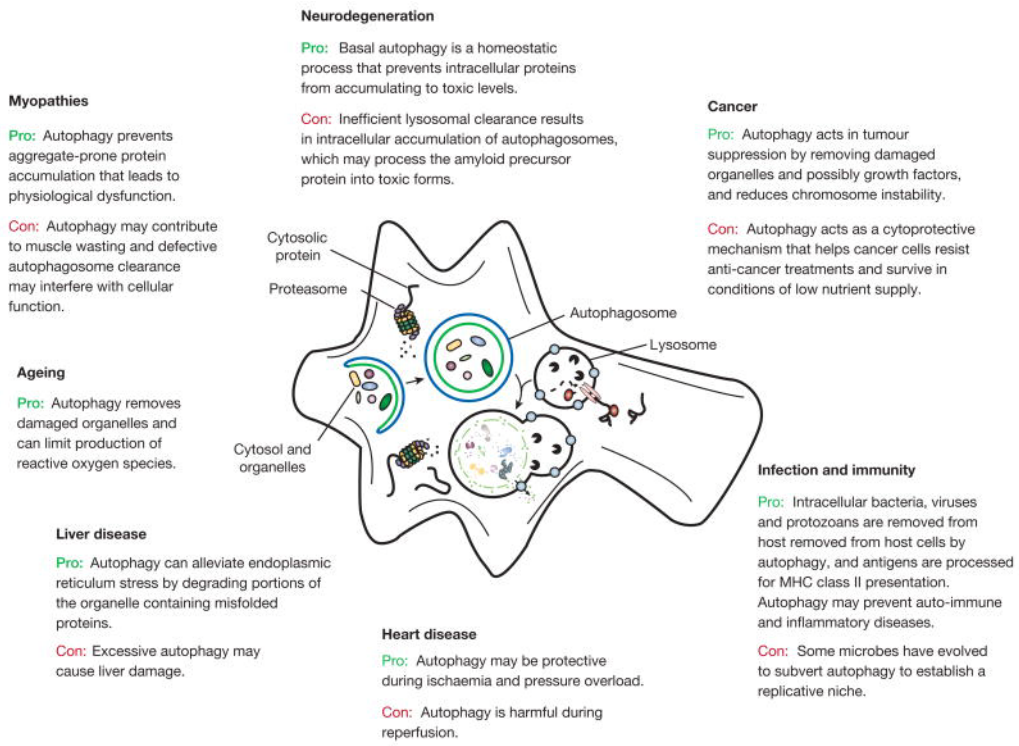


Figure 4. **Scheme resuming the role of autophagy in diseases.**
Picture taken from (Mizushima, Levine et al. 2008)

Given the importance of autophagy in the clearance of aberrant proteins, this process emerged as a potential therapeutical target for many neurodegenerative diseases. An accumulation of autophagic vacuoles in dystrophic neurites has been in fact found in the brains of AD patients (Nixon 2005; Nixon, Wegiel et al. 2005; Rubinsztein, DiFiglia et al. 2005), and mutation in PSEN1 have been proposed to play a role of defective clearance of autophagic substrates in AD patients (Lee, Yu et al. 2010). Evidence for a role of autophagy in PD are also available. Mutations in Parkin or Pink-1 are associated with an autosomal recessive form of PD and have been shown to impact the functionality of mitochondria (Exner, Treske et al. 2007; Lutz, Exner et al. 2009; Narendra, Tanaka et al. 2009; Kamp, Exner et al. 2010; Bouman, Schlierf et al. 2011): since both these proteins are active regulators of mitophagy, it has been suggested that the autophagic quality control of mitochondria may be, at least partially, responsible for the disease (Narendra, Tanaka et al. 2009; Narendra and Youle 2011; Pilsl and Winklhofer 2012).

2.2 Mitochondria: shaping the fate of cells

It is intriguing that most of the cell´s metabolism and fate relies on a bacterial relic. These double-membraned, endosymbiontic organelles are in fact known as the powerhouse of the cell due to the plethora of effects that they exert. Ranging from few hundreds to many thousands per mammalian cell, mitochondria produce most of the intracellular ATP and are involved in some of the main signaling pathways concerned with cell homeostasis, ranging from oxidative phosphorylation to redox signaling and metabolism (Knott, Perkins et al. 2008; Yen and Klionsky 2008). Moreover, they buffer the intracellular cytosolic Ca^{2+}, thus contributing to regulate Ca^{2+} signaling (Singaravelu, Nelson et al. 2011) and protecting cells from excessive Ca^{2+} influx (Knott, Perkins et al. 2008).

Despite classical studies, in which mitochondria investigated at the ultrastructural level through electron microscopy were shown as static and immobile

organelles, the advent of molecular biology and new imaging techniques revealed that mitochondria are very dynamic in their motion, and continuously undergo fission (that is, they split in two or more mitochondria) and fusion (when two mitochondria join together) events, apparently without a clear architecture (Song, Bossy et al. 2008; Mitra and Lippincott-Schwartz 2010). However, despite these apparent chaotic and random movements of mitochondria, it starts to become clear that the maintenance of a dynamic mitochondrial network is functional to the metabolic state of the cell. In particular, mitochondrial shape represents a critical aspect indicative of their health and energetic status, two features which change according to mitochondrial morphology and location within a cell, especially when the cell is polarized and possesses distinct functional subcellular compartments. For instance, in the axon of neurons mitochondria show a high degree of movement, fusion and fission, and could therefore properly reach functional spots in which energy production is particularly needed, such as synapses. As a consequence, alterations in mitochondrial functionality (which reflect the inability of mitochondria to proper move or impair the fusion-to-fission balance) are likely to participate in the progression of axonal, and thus neuronal, neurodegeneration in several diseases (Bossy-Wetzel, Barsoum et al. 2003; Bossy-Wetzel, Petrilli et al. 2008; Knott and Bossy-Wetzel 2008; Knott, Perkins et al. 2008).

2.2.1 Molecular effectors orchestrating mitochondrial dynamics

As a general principle, the morphology of mitochondria is assured by the activity of proteins mediating either mitochondrial fusion or fission: fission mainly contributes to mitochondrial renewal and redistribution (Parone, Da Cruz et al. 2008; Lackner and Nunnari 2009); Fusion is instead important for mitochondrion-mitochondrion interactions, and it promotes the exchange of metabolites and mitochondrial DNA (Scott and Youle 2010). Due to their mutual cooperation, the proteins responsible for these two mechanisms properly balance mitochondrial dynamics in response to the cell´s needs (Karbowski and Youle 2003; Anesti and Scorrano 2006; Campello, Lacalle et al. 2006; Cereghetti and Scorrano 2006). The knowledge regarding the respective mechanisms of action of these fusion and fission

proteins mostly derives from studies performed in yeast (Nunnari, Marshall et al. 1997; Shaw and Nunnari 2002; Oettinghaus, Frank et al. 2011). However, the relevant degree of conservation amongst them has made possible to correlate the results obtained in yeast with the potential mechanisms taking place in mammalian cells.

On a molecular level, all the proteins regulating mitochondrial fusion and fission belong to the family of GTPases (Fig. 5). Dynamin-related protein-1 (Drp-1) and Mitochondrial Fission Factor (Mff) are the major proteins regulating fission in mammals (Bleazard, McCaffery et al. 1999; Tieu and Nunnari 2000; Frank, Gaume et al. 2001; Osteryoung and Nunnari 2003; Szabadkai, Simoni et al. 2004; Lackner and Nunnari 2009; Otera, Wang et al. 2010): these proteins, normally located in the cellular cytoplasm, intermittently contact the outer mitochondrial membrane (OMM), possibly interacting with the outer membrane-associated protein hFIS1 (Tieu, Okreglak et al. 2002). However, the exact role of hFIS1 in recruiting Drp-1 at the OMM is still under debate, since in mammals this protein has been shown to be dispensable for the direct recruitment of Drp-1 (Lee, Jeong et al. 2004; Lee, Jeong et al. 2007).

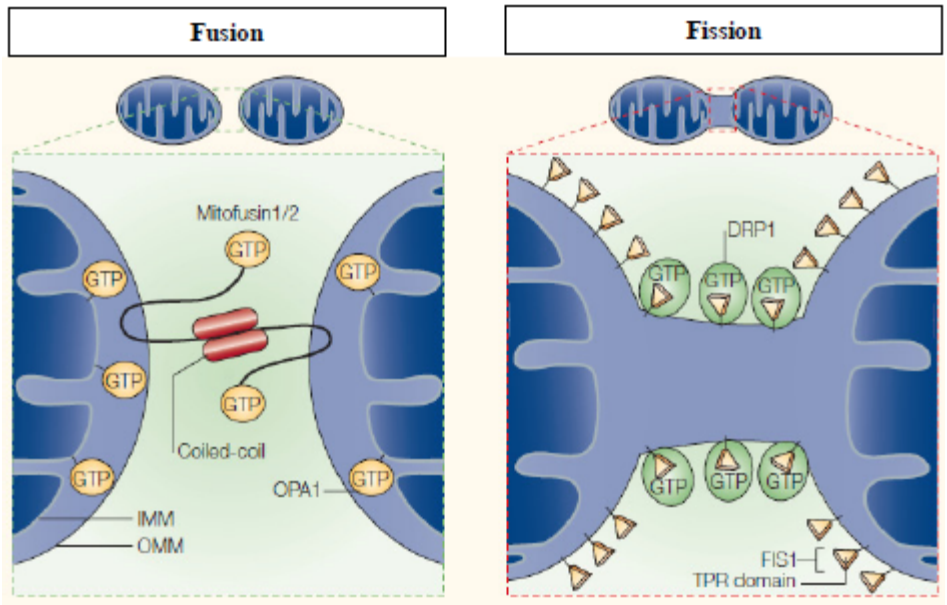


Figure 5. **The fission and fusion machinery.** Schematic representation showing mitochondrial fusion (left) and fission (right). Mitofusin 1 and 2 are responsible for fusion of OMM, while Opa1 promotes IMM fusion. Drp-1 is the major protein involved in fission.

When fission occurs, Drp-1 stably binds the OMM and there it forms clusters (foci) which identify the future division sites of the organelle (Fig. 5). During this process, mitochondria undergo massive ultrastructural changes, including degeneration of the cristae membranes, which allow the division of the organelles (Smirnova, Griparic et al. 2001). Recently, Friedman et al. (2011) demonstrated the involvement of the endoplasmic reticulum (ER) in marking the sites of division before this division occurs, suggesting a tight cooperation between fission proteins located in mitochondria and other intracellular organelles (ref) (Friedman, Lackner et al. 2011).

On the other side, mitochondrial fusion occurs via components of both the OMM and IMM (inner mitochondrial membrane). Mitofusin 1 and 2 (Mfn1, Mfn2) are two main transmembrane proteins mediating fusion at the OMM (Santel, Frank et al. 2003): it has been proposed that fusion occurs via direct tethering of the OMM of two separate mitochondria, a process involving homotypic interactions between mitofusins (Koshiba, Detmer et al. 2004). Fusion of the IMM requires instead Optic Atrophic Protein 1 (Opa1), which is located in the intermembrane space between IMM and OMM (Zanna, Ghelli et al. 2008). Opa1 can exist in up to eight different isoforms (likely having distinct functions), and the overall ratio between the short and long isoforms is believed to be regulated by proteolytic cleavage (Ehses, Raschke et al. 2009).

2.2.2 Autophagy shapes mitochondria, or the other way round

As previously described, autophagy can target mitochondria for their selective degradation and recycling of their components. This eventuality, although taking place as part of the physiological cellular metabolism, can be enhanced under specific conditions, such as when mitochondria become damaged or dysfunctional (Rambold and Lippincott-Schwartz 2011; Youle and Narendra 2011). Irrespective of

the stimulus finally leading to mitophagy, one of the first events tagging dysfunctional mitochondria is represented by a depolarization of their membrane. Such depolarization can in turn be recognized by the voltage sensitive Pten-Induced Putative Kinase 1 (Pink1) (Jin, Lazarou et al. 2010). Normally, Pink1 is subjected to high turnover rates in mitochondria, however upon loss of mitochondrial potential this protein becomes stabilized on the OMM, thus facilitating the subsequent recruitment of Parkin, an E3-ubiquitin ligase responsible for the ubiquitylation of several mitochondrial proteins such as Mfn1, Mfn2 and the Voltage-Dependent Anion Channel protein VDAC (Jin, Lazarou et al. 2010; Narendra, Kane et al. 2010; Narendra, Jin et al. 2010; Karbowski and Youle 2011; Narendra and Youle 2011; Youle and Narendra 2011; Pilsl and Winklhofer 2012). Ubiquitylation represents indeed the last step before autophagosome formation and degradation of mitochondria, which occurs through the recruitment of other proteins such as p62 (Ichimura and Komatsu 2010; Komatsu and Ichimura 2010; Isogai, Morimoto et al. 2011). In addition, several other components regulate mitophagy. Amongst these are NIX (Aerbajinai, Giattina et al. 2003; Dorn 2010; Kanki 2010; Kanki and Klionsky 2010), Ambra1 (Fimia, Stoykova et al. 2007; Herrera, Decano et al. 2009; Di Bartolomeo, Corazzari et al. 2010; Strappazzon, Vietri-Rudan et al. 2011)and essential proteins of the autophagic machinery like Atg7 and Atg5 (Komatsu, Waguri et al. 2005; Stephenson, Miller et al. 2009; Vazquez, Arroba et al. 2012).

Despite the described mechanism of mitophagy, two recent papers have now suggested that mitochondria can be spared from autophagy through a mechanism which implies their hyperelongation. In the first of these studies, Gomes and coworkers (Gomes, Di Benedetto et al. 2011) showed that, under starvation, mitochondria react by increasing their degree of fusion, a finding quickly supported from a second work (Rambold, Kostelecky et al. 2011). The resulting network of hyperelongated (or hyperfused) mitochondria was shown to depend upon the inhibition of Drp1, and this mechanism was ultimately necessary to spare these organelles from the otherwise obligatory autophagic pathway. Functionally, this new mechanisms through which cells could maintain their mitochondrial network in critical conditions of starvation justifies the fact that ATP production could be

preserved and cell death avoided (Gomes, Di Benedetto et al. 2011). However, it remains to understand if hyperfusion represents one last tentative of starving cells to delay their death or rather if it is a transient condition that cells only use in extreme cases, such as when facing a reduction of nutrients, after which the regular mitochondrial network could be restored.

Recent findings have drastically changed the view that autophagy and mitochondria are linked each other uniquely by mitophagy. Remarkably, one study has now pointed out an interesting aspect of the crosstalk between autophagy and mitochondria, suggesting that these organelles do not exclusively represent a substrate for autophagic-mediated renewal but, rather, are capable to independently promote the formation of new autophagosomes (Hailey, Rambold et al. 2010), a mechanism previously believed to occur only through membrane supply from the ER (Axe, Walker et al. 2008), Golgi (Young, Chan et al. 2006) and plasma membrane (Ravikumar, Moreau et al. 2010). In the work of Hailey et al, mitochondrial membrane is shown to be the primary site of the production of phosphatidylethanolamine during starvation-induced autophagy, different from normal conditions, in which is rather the ER providing this autophagic precursor through the Kennedy reaction (McMaster and Bell 1997). Nutrient depletion would then be the discriminating factor by which the cells switch from ER to the mitochondria for producing autophagosomes.

Mitochondrial redox signaling is an additional way that links mitochondria with changes in the autophagic activity of cells. It has been reported that byproducts of the oxidative metabolism, such as ROS and RNS, are able to stimulate autophagy (Murphy 2009). For instance, it has been shown that H_2O_2 can oxidize mitochondrial proteins, thus impairing the electron-transfer process and thereby inducing the generation of superoxide (Beckman 2002). Although low levels of ROS play important roles in cell signaling, elevated levels can severely damage the functionality of mitochondria by oxidizing proteins and lipids, resulting in a detrimental output for the cell. On the other hand, RNS, and in particular those derived by nitric oxide (NO), have been described to exert a variety of effects on autophagy. Barsoum and colleagues (2006) reported that NO-donors are able to

induce Drp-1 dependent fission of mitochondria in primary cortical neurons (Barsoum, Yuan et al. 2006). Moreover, studies performed in different cell lines showed that NO is able to inhibit IKKb and JNK1, leading to the activation of Beclin1 and to the promotion of the autophagic response (Sarkar, Korolchuk et al. 2011).

3 Specific aims

In order to understand the mechanisms controlling the reactivity of astrocytes during brain inflammation, in particular which of the cellular processes are among the first to become altered, we investigated the mitochondrial dynamics in primary astrocytic cultures following acute inflammation. Confocal microscopy, fluorescent probes and live-imaging were used to monitor the influence of pro-inflammatory mediators on mitochondrial morphology, motility and energetic status. Alterations in mitochondrial dynamics were tracked at the level of single organelles and linked to the activation of autophagy in inflamed astrocytes. Furthermore, co-cultures of cortical neurons and astrocytes were used to assess the cell-specific contribution of this subpopulation of glial cells in sustaining or impairing neuronal survival under inflammation.

RESULTS

1.1. Astrocytes respond to pro-inflammatory molecules by transiently rearranging their mitochondria

To investigate if the onset and progression of an inflammatory reaction could affect the general viability of astroglial cells, we established an *in vitro* model which mimics the instauration the inflammatory process. To this aim, we made use of primary cultures of cortical astrocytes from P1-P2 mice, in which the superficial layers of the somatosensory and motor cortex were dissociated and cells were plated and mantained in DMEM + 10% FCS medium until reaching confluency (usually 2-3 weeks), as previosly described (McCarthy and de Vellis 1980). The enrichment in astrocytes of these cultures (>85%) was then confirmed by performing immunostaining for typical astrocytic markers, such as the glial-fibrillary acidic protein (GFAP) (Fig. 6A-B) or the calcium-binding protein S100β. To mimic an inflammatory environment, we have chosen to use a combination of lipopolysaccharide (LPS; 1 μg/μl) and interferon-γ (IFN-γ; 1 μg/μl), hereafter defined as LI, because of the well known capacity of these molecules to induce a strong and reliable inflammatory response in glial cells (Bal-Price and Brown 2001). As one of the main aims of this thesis is to unravel the initial events undergoing reactivity in astrocytes following inflammation, we then restricted the analysis to the first 1-2 days following LI treatment. First, we performed a cytofluorimetric assay with fluorescein labeled Annexin V as a read-out of ongoing apoptosis and cell death in inflamed astrocytes. No differences in apoptosis or cell death were observed between control or inflamed astrocytes (Fig. 6C), suggesting that during this short period upon LI treatment, no significant alteration in cell viability could be detected. Then, we investigated the intracellular production of ATP by chemiluminescence, and observed about a 2-fold increase of ATP production following inflammation already starting from 2 hours post-LI treatment (Fig. 6F), indicating that astrocytes rapidly underwent a metabolic change which implies the request of more energy. To investigate the intracellular origin of such increase in ATP production, we repeated the same experiment under low-glucose conditions, an experimental paradigm often

used to discriminate between mitochondrial and glycolitic ATP-production. Interestingly, in low-glucose medium the levels of produced ATP were indistinguishable between control and inflamed astrocyes at all examined time-points (Fig. 6F), suggesting that the glycolitic pathway takes over in producing ATP during stimulation of astrocytes with pro-inflammatory molecules.

To better understand if mitochondria could be directly involved in the cellular changes initiated by inflammation, we performed a time-course of mitochondria analysis upon LI treatment and investigated the morphology of mitochondria by labeling astrocytes with MitoTracker Red. Surprisingly, we could observe a stereotipic pattern of progressive morphological alterations in mitochondria following inflammation, which consisted in the formation of either globular or rod-like structures, very dissimilar from the tubular structures typical of healthy astrocytes (Fig. 6D). The appearance of this phenotype started as early as 4 hours post-LI treatment, with the extremities of tubular mitochondria rounding up to form loop-like structures, it peaked by 8-12 hours and started to disappear by 24 hours post-LI treatment, with astrocytes showing again a tubular mitochondrial population and few fragmented or rod-like mitochondria (Fig. 6D). We also quantified the mitochondrial length of the astrocytes during this time-window (Fig. 6E), and we found out that mitochondria become shorter over the time, reaching up to one third of the usual length of control mitochondria at 8 hours (51% Ctrl mitochondria: >3 μm; 48% LI: 1-2 μm).

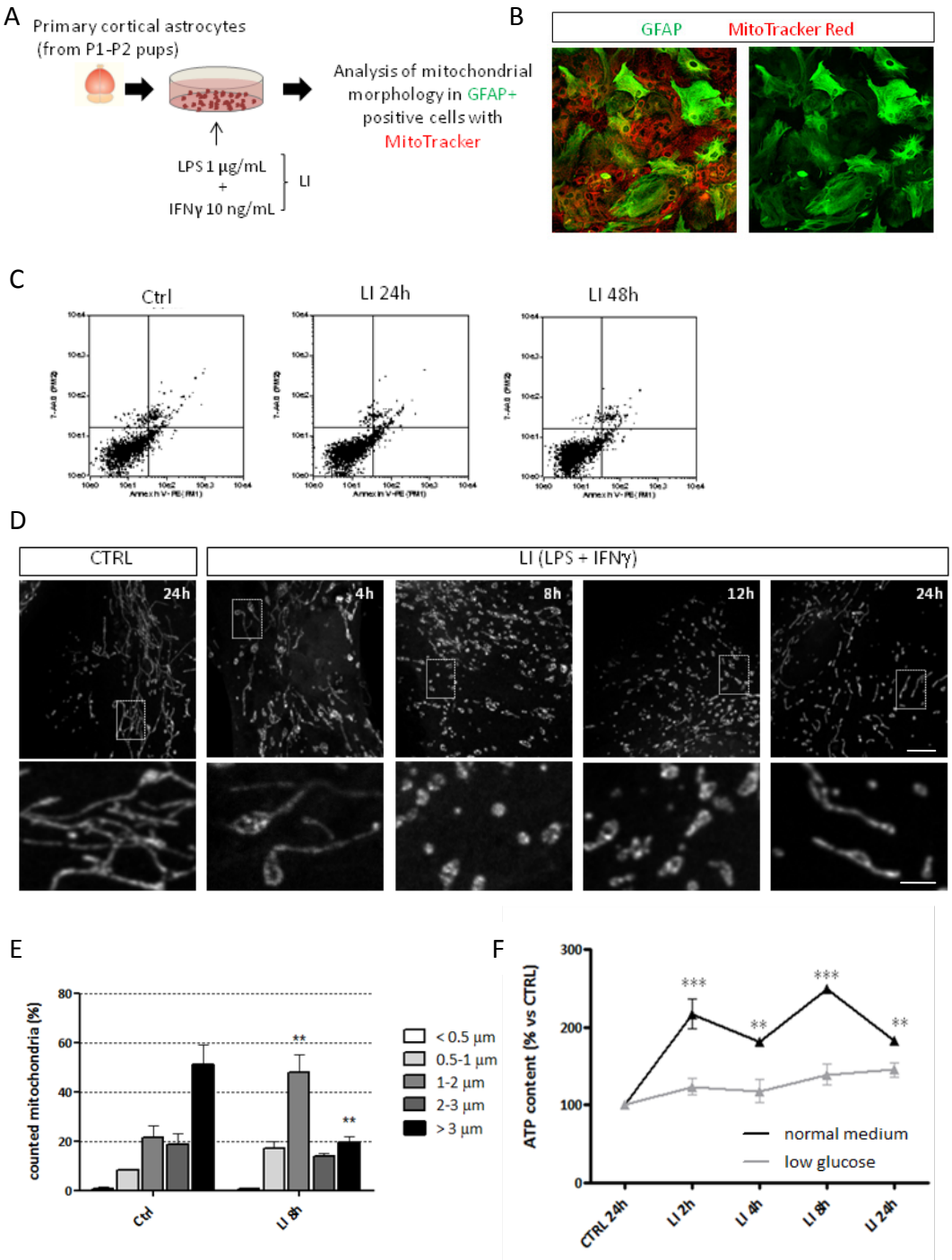


Figure 6. **Astrocytes respond to pro-inflammatory molecules by transiently rearranging their mitochondria.** ***(A)*** Schematic representation of the astrocytes culture preparation and treatment. ***(B)*** Immunostaining of astrocytes for GFAP (green), a marker commonly used to label astrocytes, and MitoTracker Red (red), to label mitochondria. ***(C)*** Cytofluorimetric analysis of apoptosis and necrosis with Annexin-V and 7-AAD of Ctrl and LI-treated astrocytes. ***(D)*** High resolution confocal images of Ctrl and LI-treated astrocytes at the given time-points. Astrocytes were previously incubated with

MitoTracker Red to visualize mitochondria. Magnification of selected regions of the cells (dashed squares) are shown below each panel. Bars, 5 and 2 μm. ***(E)*** Quantification of mitochondrial length using ImageJ, as reported in Materials&Methods. **p< 0.01 versus Ctrl mitochondria. ***(F)*** ATP measurements of Ctrl and LI-treated astrocytes in the presence of normal medium (black line) or low glucose medium (grey line). **p< 0.01 versus Ctrl, ***p< 0.001 versus Ctrl.

1.2. Inflammation-mediated changes of mitochondrial dynamics entail reduced motility, fragmentation and clustering

Given the characteristic change in mitochondrial morphology observed after inflammation, which anyway appeared to be confined to the first hours after LI treatment, we performed a set of live imaging experiments to study the progressive alteration of the mitochondrial network. Time-lapse experiments were performed at controlled temperature (37°C) and CO_2 (5%) up to 8-12 hours by imaging single astrocytes previously transfected with a plasmid encoding for mito-GFP, in which the GFP fluorochrome is selectively targeted to mitochondria through its fusion with the subunit VIII of the cytochrome-C oxidase. Imaging of these transfected astrocytes allowed us to monitor in real-time the time-dependent alterations in mitochondrial dynamics, otherwise impossible to presume from experiments conducted in fixed cells. In most movies, control cells display a higly dynamic, but homogeneous network of mitochondria throughout the recording. Differently, inflammation induces the progressive alteration in the distribution of mitochondria, followed by the terminal clustering of some mitochondria around the perinuclear region 8-12 hours post-treatment (data not shown).

Time-lapse video imaging revealed that the earliest events underlining mitochondrial dynamics following inflammation could be a change in mitochondrial motility. To quantify this mitochondrial motility, we acquired z-stack confocal frames at high frequency (1 frame every 30 seconds), in order to effectively track single mitochondria across time (Fig. 7A). Fig. 7B shows a sequence of representative frames, spaced 4 minutes each, in a region of interest within a control or inflamed astrocyte at 4 hours after LI treatment. Single mitochondria (two mitochondria in the control astrocyte and four mitochondria in the treated astrocyte) were identified off-line and pseudo-colored in both cells, to facilitate the analysis.

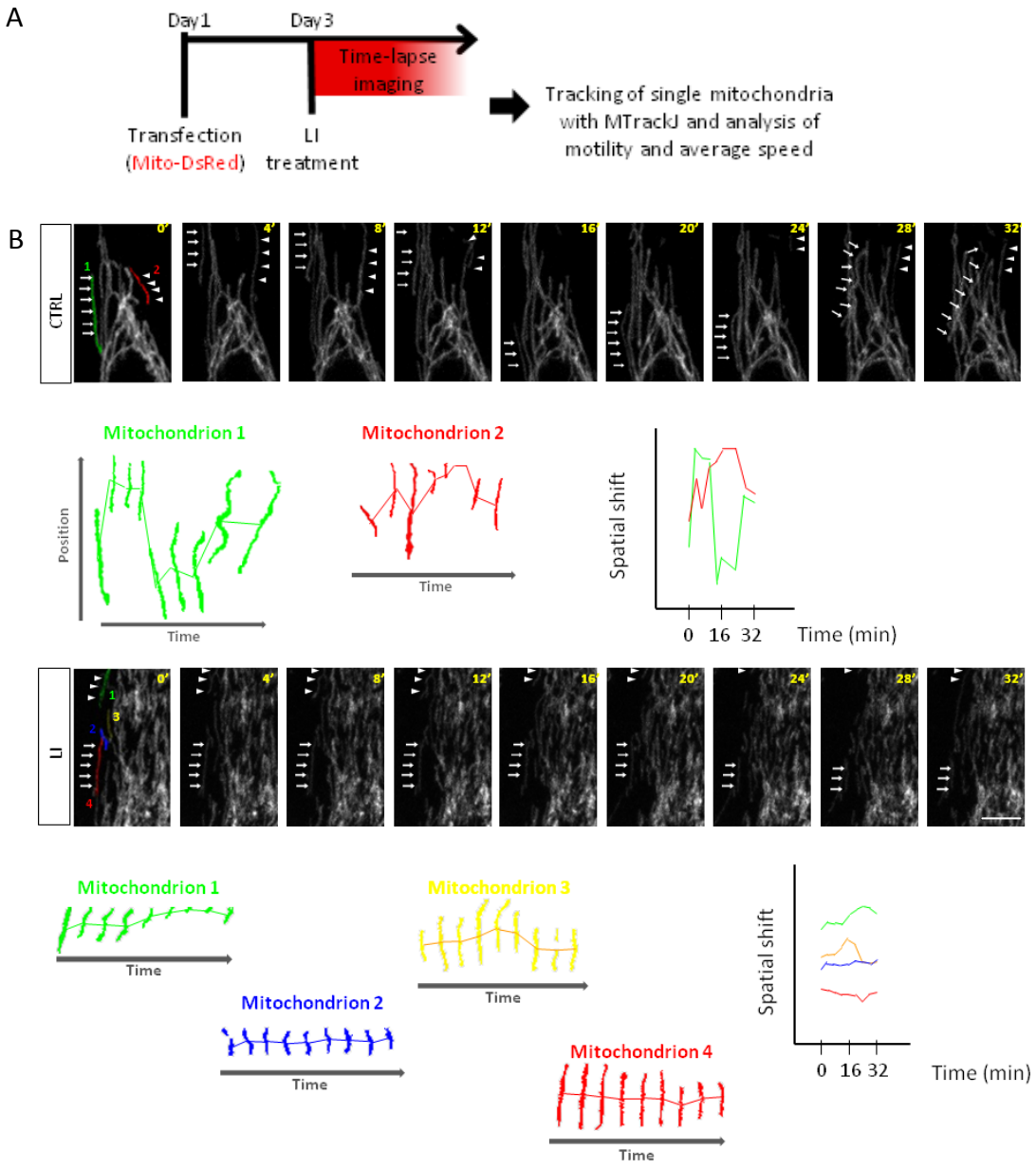


Figure 7. **Tracking of mitochondria by live-imaging microscopy.** ***(A)*** Scheme resuming the experimental plan for mitochondrial tracking. ***(B)*** Representative frames from movies taken on either Ctrl or LI-treated astrocytes, previously transfected with mito-GFP. Representative tracked mitochondria are drawn off-line in pseudo-colors (two for Ctrl astrocytes and four for LI-treated astrocytes) and qualitative analysis of the relative spatial shift is shown. Bar, 5 μm.

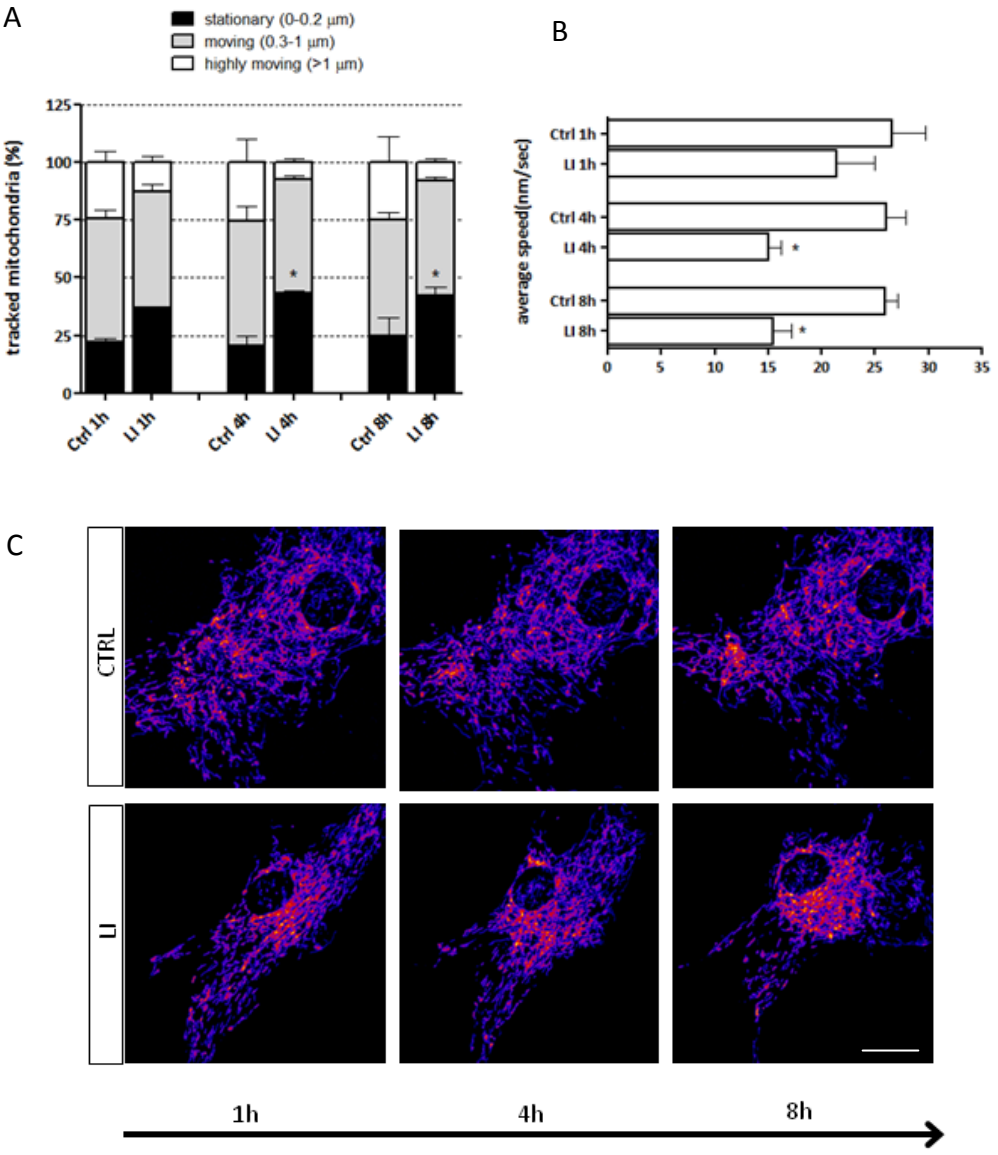


Figure 8*. Inflammation-mediated changes of mitochondrial dynamics entail reduced motility, fragmentation and clustering.** ***(A) Quantification of mitochondrial motility upon inflammation with MTrackJ, as reported in Materials&Methods. Mitochondria were classified in three different groups according to the D2P value. *p< 0.05 versus Ctrl. ***(B)*** Average speed of tracked mitochondria using MTrackJ. *p< 0.05 versus Ctrl. ***(C)*** Representative frames taken from movies of either Ctrl or LI-treated astrocytes previously transfected with mito-GFP, that show the progressive clustering of mitochondria to the perinucleus. To highlight the perinuclear clustering of mitochondria, images were false-coloured. Bar, 10 µm.

Whereas control cells displayed high degree of motility during the imaged time window, which could be appreciated by the spatial shift of these individual mitochondria across frames, inflamed astrocytes showed shorter and much more static mitochondria, the motility of which appeared drastically reduced compared to control mitochondria as indicated by their spatial shift analysis (Fig. 7B). Quantification of mitochondria motility at 1, 4 and 8 hours post-treatment resulted in the graph showed in Fig. 8A, in which the relative motility amongst mitochondria was divided in 3 different classes, depending on the mean D2P value (see Materials and Methods for details): stationary (D2P <0,2 μm), moving (D2P 0,3-1 μm) and highly moving (D2P >1 μm). Compared to control cells, treated astrocytes displayed a higher percentage of stationary mitochondria already 1 hour after inflammation, indicating that these are higly sensitive organelles which rapidly respond to the presence of pro-inflammatory molecules. Notably, this difference significantly increased by 4 and 8 hours post-LI treatment, concomitantly with a reduction of the proportion of highly moving mitochondria (Fig. 8A). According to these quantifications, the average speed of mitochondria at these time-points progressively decreased in inflamed astrocytes (Fig. 8B). Remarkably, the observed changes in mitochondrial motility and length correlated with the general distribution of mitochondria in treated astrocytes, with a substantial fraction of them becoming clustered around the perinucleus (Fig. 8C). Together, these data suggest that inflammation rapidly induces a temporal sequence of changes in mitochondrial dynamics occurring within few hours and involving (i) mitochondrial motility, (ii) average mitochondrial speed and (iii) clustering of mitochondria.

1.3. Fusion is impaired in the initial phases of mitochondrial rearrangement

A proper balance of fusion and fission represents an important mechanism in the omeostatic maintenance of the mitochondrial network in a living cell (Scott, Cassidy-Stone et al. 2003; Suen, Norris et al. 2008). For this reason, and given the above described key observations demonstrating an altered mitochondrial motility occurring quickly upon inflammation, we monitored the fusion proficiency of mitochondria following inflammation. To this aim, astrocytes were transfected with a plasmid encoding for the photoactivatable(PA)-mitoGFP, a fluorophore that is targeted to mitochondria but that becomes detectable in the GFP spectrum exclusively after its irradiation with wavelengths in the UV spectrum (Karbowski, Arnoult et al. 2004). In addition to this construct, astrocytes were transfected with the mito-DsRed plasmid, in order to selectively direct the UV laser beam onto a well defined population of mitochondria within the cell. As illustrated in Fig. 9A, the photo-activation of an irradiated squared area (in green) mediates the conversion of the PA-mitoGFP, which then becomes detectable with standard GFP filters. Thus, time-lapse imaging of GFP-emitting photo-activated mitochondria and DsRed-emitting mitochondria results in a direct measurement of the fusion events of the photo-activated mitochondria, through merging of the GFP and DsRed signals. Fig. 9 B shows the time-course of fusion events after photo-activation of a defined square area (green square in the cell overview) across 40 minutes, in which the cells were imaged every 5 minutes. The sequence depicted in the upper panels shows the progressive diffusion of the GFP signal from the original ROI of photo-activation to the non-photo-activated external area in a control astrocyte. The first and last frames are presented as merge of the GFP (photo-activated mitochondria) and DsRed (non-irradiated mitochondria) channels showing that, over time, green mitochondria fuse with red mitochondria (yellow signal) just beyond the borders of the ROI in a control cell. In the lower panels, an inflamed cell was subjected to the same analysis: in this case, the GFP signal almost did not diffuse out of the ROI of irradiation, indicating that mitochondrial fusion with DsRed-expressing mitochondria did not occur. Quantification of 4 cells per experimental condition (control vs. inflamed at 4 hours

post-LI treatment) resulted in a progressive decrease in the signal intensity of the GFP within the ROI in control, but not in inflamed astrocytes (Fig. 9C). This reduction of the GFP signal observed in control cells indicates that mitochondria underwent more fusion events than inflamed cells.

Moreover, the analysis of an outer ROI (Fig. 4D-E), corresponding to the concentric area to the initial ROI of photo-activation, shows that the GFP signal significantly increases in this area over the time in control cells, in marked contrast to inflamed cells, again demonstrating that fusion did not occur upon inflammation (Fig. 9D-E). Accordingly, the comparison between the GFP signal appearing in the outer ROI and that of a reference ROI located far from the site of photo-activation, confirmed this data.

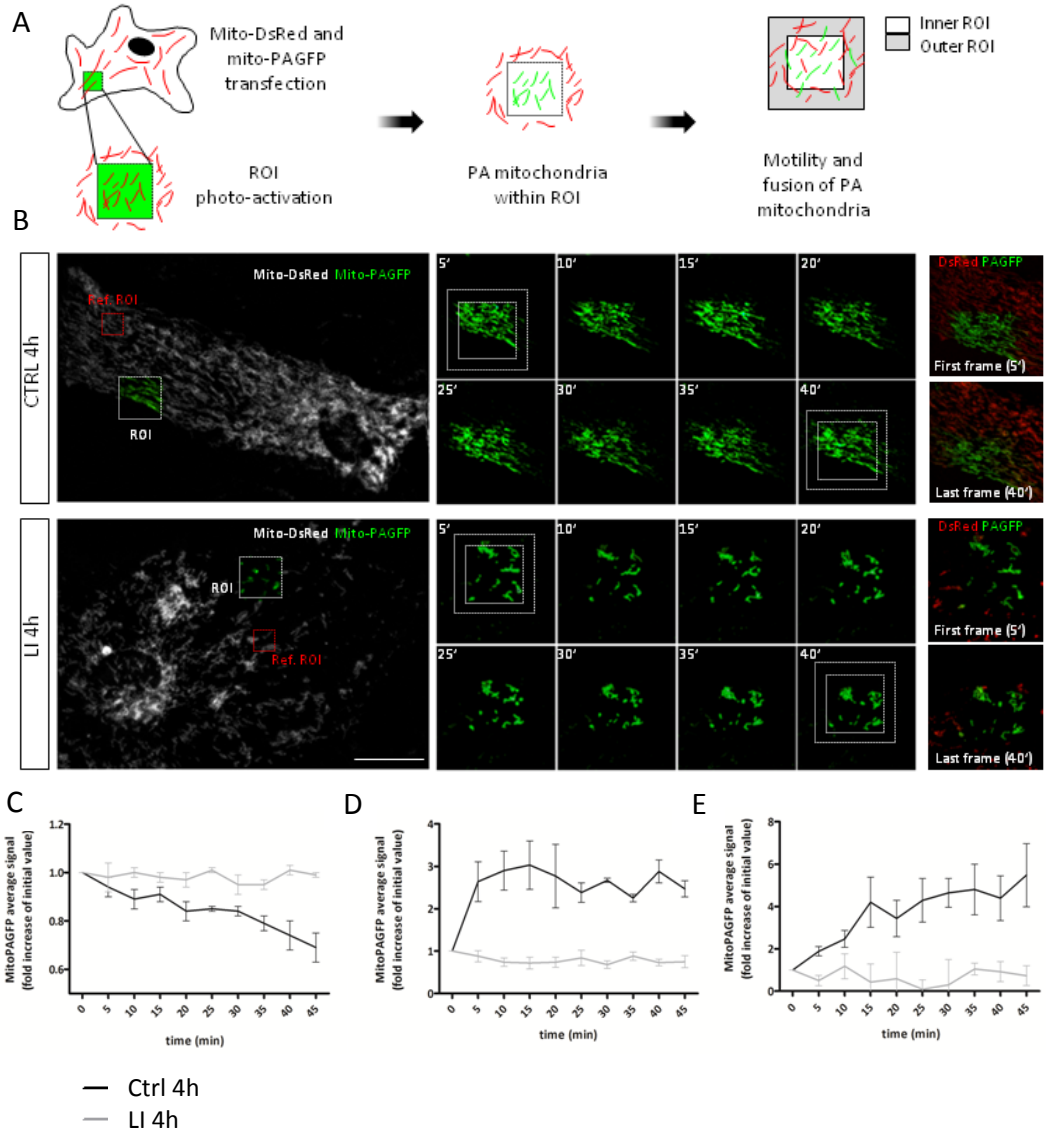


Figure 9. **Fusion is impaired in the initial phases of mitochondrial rearrangement.** ***(A)*** Schematic representation showing the photoactivation of a specific region of interest (ROI), depicted in green in the left panel; definition of an inner ROI (white square, right panel) and an outer ROI (grey square, right panel), used for the following analysis. ***(B)*** Representative frames taken from movies of astrocytes previously transfected with mito-DsRed and mito-PAGFP. The inner and outer ROI of the given cells are in green and red, respectively. For every condition, the first and the last merged frames of the movies are shown (right panel). Fusing mitochondria become yellow. Changes in fluorescence intensities of the inner ROI ***(C)***, outer ROI ***(D)*** and the ratio inner/outer ROI ***(E)*** were also quantified over the time. Bar, 10 μm.

1.4. Role played by nitric oxide in mitochondria remodeling during inflammation

It is well known that inflammatory stimuli induce iNOS up-regulation in glial cells, with the consequent increase in NO production (Almeida, Almeida et al. 2001). Accordingly, astrocytes showed a rapid up-regulation of iNOS following LI tratment, which was evident already by 4h later (Fig. 6A). To effectively demonstrate that NO was produced under LI tratment and to increase the temporal resolution of our measurments, we performed a detailed analysis of NO production over the time by using the DAF-FM indicator. In these experiments, a positive control consisting in the stimulation of astrocytes with 100 μM SNAP, a NO donour, was used. Indeed, SNAP promptly released NO in the astrocytic cultures, which accumulated over time and was detected by the assay (Fig. 10B). LI stimulation induced a similar increase in the intracellular NO concentration already starting from 30 min post-treatment: the amount of NO did not further increase, but rather stayed stable for the entire course of the experiment (Fig. 10B), indicating that a constant production of NO characterizes inflamed astrocytes during the first 24 hours of stimulation. Since iNOS up-regulation and NO production represent molecular hallmarks of an ongoing process of inflammation and are readily reproduced in astrocytes, we asked whether the observed mitochondrial rearrangement was somewhat dependent of the induction of this enzyme. To this aim, we pre-treated astrocytes with L-NAME, a well known inhibitor of iNOS, before inducing inflammation. Remarkably, mitochondria appeared to be completely rescued in the morphology following inflammation, as shown in Fig. 10C-D, in which astrocytes labeled with MitoTracker Red exhibited a tubular morphology of mitochondria only in the presence of L-NAME. These results indicate that the mitochondrial rearrangement observed following LI treatment requires NO to occur, and interfering with iNOS may prevent inflammation-dependent alteration of mitochondria.

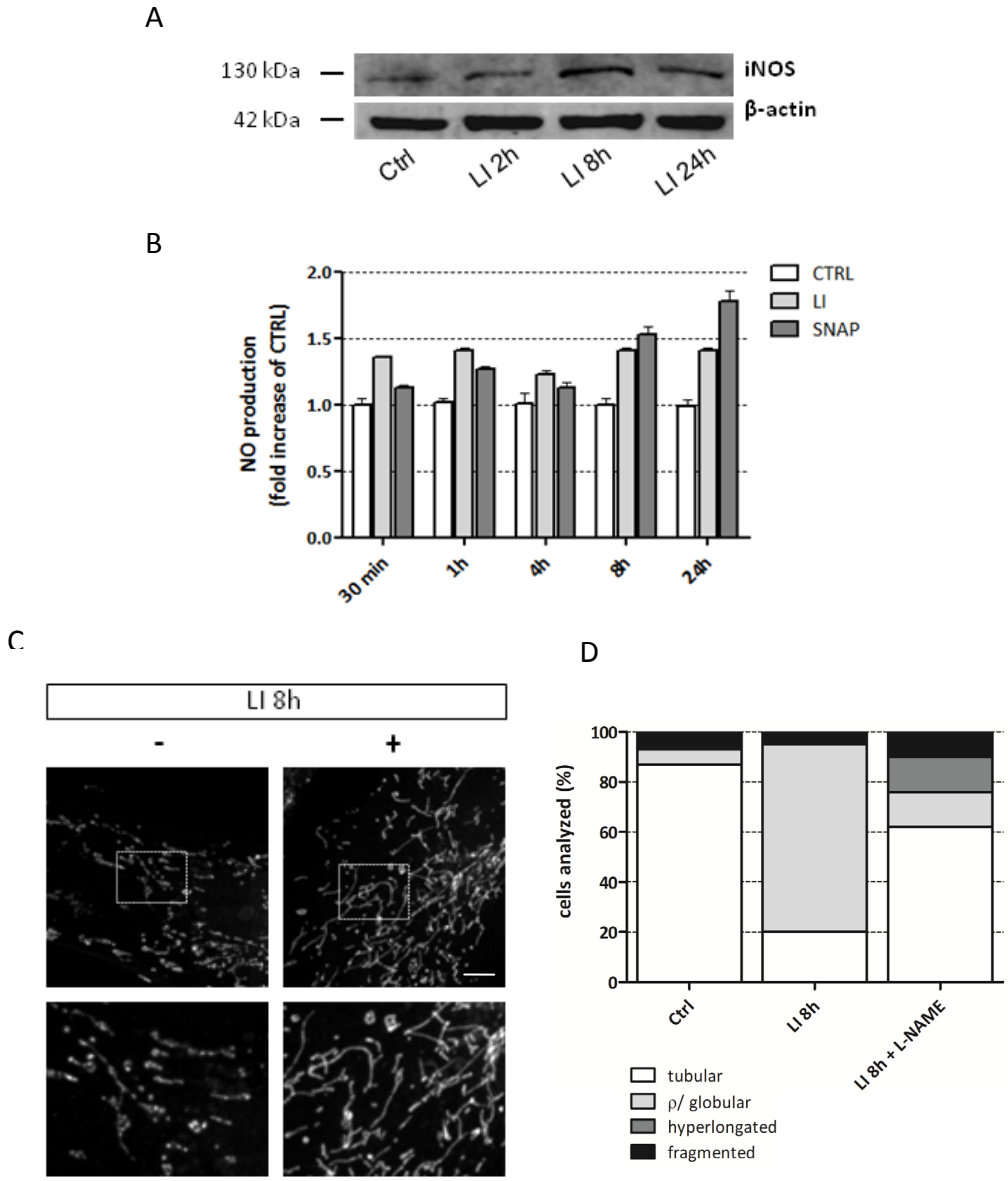


Figure 10. **Role played by nitric oxide in mitochondria remodeling during inflammation.** ***(A)*** Time-course of iNOS induction upon LI treatment by Western Blot. ***(B)*** Quantification of NO production with DAF-FM following LI o application of the NO donor SNAP. ***(C)*** High resolution confocal images of MitoTracker Red-treated and LI-treated astrocytes in the absence or presence of the iNOS inhibitor L-NAME and ***(D)*** relative quantification of mitochondrial morphology. Magnifications of selected regions of the cells (dashed squares) are shown below each panel. Note the abolishment of mitochondrial rearrangement in inflamed astrocytes when pretreated with L-NAME. Bar, 5 μm.

1.5. Altered mitochondria produce high levels of ROS

In mammalian cells, mitochondria represent one of the major sources of ROS, which are by-products of the oxidative respiration under basal conditions. When mitochondria are damaged, the consequent dysregulation of the oxidative phosphorylation machinery results in an increased generation of ROS (Brookes, Yoon et al. 2004). We thus tested whether, during inflammation, mitochondria display an altered ROS production in comparison with mitochondria of control astrocytes. To verify this hypothesis, we co-stained mitochondria of control and LI-treated astrocytes with MitoTracker Green, to visualize the overall mitochondrial population, and with MitoSOX, a rhodamine derivative that selectively binds mitochondrially-derived superoxide molecules. we checked the mitochondrial ROS (mROS) production 24h after the induction of inflammation, the time point in which only a small portion of mitochondria maintains an altered morphology. As illustrated in Fig. 11, mROS production was nearly undetectable in mitochondria of control astrocytes, in which the dominant phenotype was tubular. We also observed nuclear unspecific staining (N) of MitoSOX, since this tracker has a hydroethidine residue that can stain nuclei as well. Interestingly, inflamed astrocytes showed a different pattern of mROS reactivity, depending on the mitochondrial morphology: whereas the tubular network displayed very little staining for MitoSOX, the few fragmented mitochondria still present in the periphery of the cells exhibited a clear upregulation of mROS levels.

These data strongly suggest that ROS production at the level of individual mitochondria correlates with their morphology. In turn, this could indicate that inflammation-triggered intracellular cascades mediate the damage of mitochondria and the over-production of ROS.

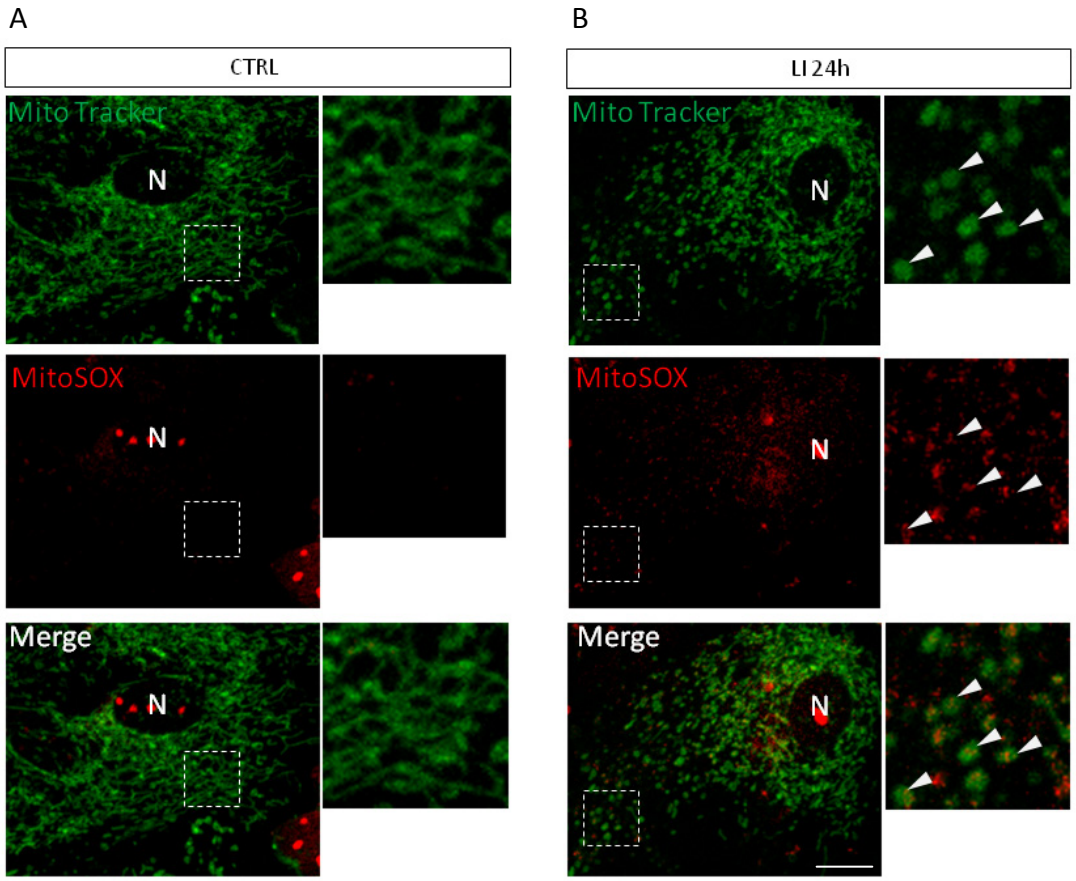


Figure 11. **Altered mitochondria produce high levels of ROS.** ***(A)*** Representative pictures (single and merged channels) of cultured astrocytes incubated with MitoTracker Green and MitoSOX, a dye specific for labeling mitochondrial-derived superoxide, and kept in control ***(A)*** or inflamed conditions ***(B)***. Magnifications of specific regions (dashed squares) are shown on the right of each picture. Unspecific nuclear staining of MitoSOX is marked with N. Damaged mitochondria in LI-treated astrocytes showed higher immunoreactivity for MitoSOX (arrowheads). Bar, 10 μm.

1.6. Autophagy is a key feature of inflamed astrocytes

Mitophagy is a well known mechanism adopted by the cell for assuring a proper mitochondrial quality control. As previously illustrated, astrocytic mitochondria undergo fragmentation and exhibit high levels of ROS production following pro-inflammatory stimuli. We then postulated that, during the first 24 hours of inflammation, mitophagy could play a critical role in eliminating damaged mitochondria, thus contributing to the re-establishment of a proper tubular network observed after 24h (Fig. 6). To check for this possibility, We performed time-lapse experiments by co-transfecting astrocytes with mitoDsRed in which, similarly to mitoGFP, the DsRed fluorochrome is selectively targeted to mitochondria, and LC3-GFP, a fusion protein between the microtubule-associated protein LC3 and the GFP fluorochrome which allows for monitoring the autophagosome formation (Bampton, Goemans et al. 2005; Klionsky, Abeliovich et al. 2008). We then imaged control and inflamed astrocytes up to 12 hours, with an acquisition rate of one frame every 4 minutes. Fig. 12 depicts representative frames extracted at key time-points from a recorded movie. Under basal conditions, control astrocytes maintained a stable tubular mitochondrial network throughout the entire recording (Fig. 12, upper panel), whereas the LC3-GFP expression pattern was mostly diffused in the cytosol, with the formation of very few GFP punctae (indicative of autophagosomes) which number was stable over the time. On the contrary, upon inflammatory stimuli the mitochondrial population showed a progressive clustering (Fig. 12, lower panel), paralleled by the generation of numerous punctae of LC3-GFP, typical of autophagy induction (Klionsky, Abeliovich et al. 2008). Colocalization analysis between DsRed (mitochondria) and GFP (autophagosomes) revealed that the formation of autophagosomes structures preferentially occurred at sites of mitochondrial clustering (Fig. 12, magnifications). Thus, inflamed astrocytes respond with mitochondrial rerrangement and, in parallel, undergo autophagy.

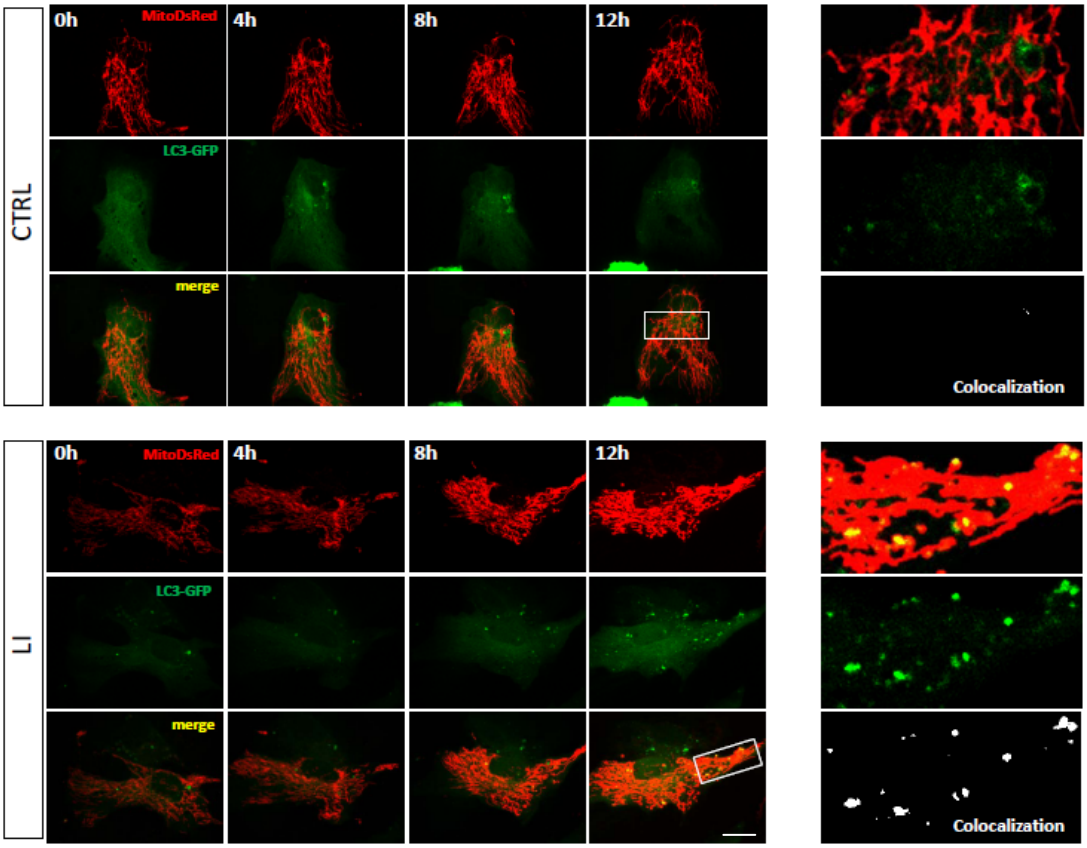


Figure 12. **Autophagy is a key feature of inflamed astrocytes.** Representative frames of 12h movies taken from cultured previously transfected with MitoDsRed and LC3-GFP. Inflammation induces a time-dependent formation of autophagosomes, that goes in parallel with the mitochondrial clustering (lower panel). The right panel shows the colocalization degree between the signals coming from MitoDsRed and LC3-GFP. Bar, 10 μm.

1.7. Autophagic markers co-localize with fragmented mitochondria: mitochondria are fated to mitophagy during inflammation

To investigate in detail the relationship between the formation of inflammation-induced autophagosomes and the morphological changes observed in mitochondria, we first analyzed the time-dependent expression of the endogenous levels of LC3B-II, the LC3B isoform known to be responsible for the formation of the autophagosome (Mizushima and Yoshimori 2007). Western blot performed on confluent cultures of astrocytes treated with LI for different time-points revealed the abundant conversion of LC3B-I into LC3B-II starting 4 hours after tratment, peaking at 8 hours and declining at 24 hours (Fig. 13). Interestingly, LPS treatment *per se* was able to elicit a similar increase in LC3B-II lipidation (Fig. 13B). To analyze the cellular distribution of the newly-formed autophagosomes with respect to the mitochondrial morphology after inflammation, we performed an immunostaining for LC3B-II at the time of its highest expression, 8 hours post-treatment. To this aim, control or LI-treated astrocytes were labeled with MitoTracker Red immediately before fixation and astrocytes were processed by immunocytochemistry with an antibody specific for LC3B-II. In control cells, tubular mitochondria were devoid of any LC3B-II immunoreactivity, which was found to be low in intensity and homogenously distributed within individual cells (Fig. 13A, left panel). On the contrary, inflammation induced a considerable increase in the immunoreactivity of LC3B-II, which mostly colocalized with fragmented and rod-like mitochondria (Fig. 13A). Three-dimensional reconstruction of the acquired images revealed the high degree of colocalization between altered mitochondria and LC3II-B (Fig. 13A, right panel), suggesting that damaged rod-like mitochondria may be preferentially fated to degradation via autophagosomes and thus subsequent fusion with lysosomes (He and Klionsky 2009) or that they could selectively promote the formation of autophagosomes themseves, as the external mitochondrial membrane has been recently proposed to contribute in the genesis of new autophagosomes (Hailey, Rambold et al. 2010). If altered mitochondria would be finally targeted to lysosomes, we hypothesized that a an immunostaining for a specific lysosomal marker such as

Lamp-2 (Bampton, Goemans et al. 2005) could reveal this phaenomenon. Thus, we transfected astrocytes with mito-GFP, to reveal the mitochondrial network, and then checked whether the LC3B-II positive mitochondrial structures were also colocalizing with Lamp-2. Fig. 14 shows the immunocytochemistry of control and inflamed astrocytes at 4 and 24 hours post-treatment. In control cells, no overt colocalization between mito-GFP, LC3B-II and Lamp-2 was observed. Upon inflammation, and following mitochondrial rearrangement, the levels of LC3B-II increased and co-localized with mito-GFP; however, albeit mitochondria and LC3B-II were closely apposed with lysosomes, no evident co-localization with Lamp-2 was observed at this time-point (Fig. 14A). On the other hand, a clear colocalization of Lamp-2 and LC3B-II was found 24 hours after LI stimulation (Fig.14A) suggesting that, during the course of the experiment, mitochondria first enucleated within autophagosomes and only later some of these mitochondria-autophagosome complexes were targeted to the lysosomal pathway. Interestingly, the effect induced by LI stimulation on mitochondrial rearrangement and autophagosome formation could be considered as a general mechanism adopted by astrocytes to react to inflammation: stimulation with IL-1β, IL-6 and TNF-α, all cytokines that are physiologically released during the inflammatory response, produced a comparable effect to that of LI treatment on LC3B lipidation when this was assessed by immunostaining (Fig. 14A) or western blot (Fig. 14B).

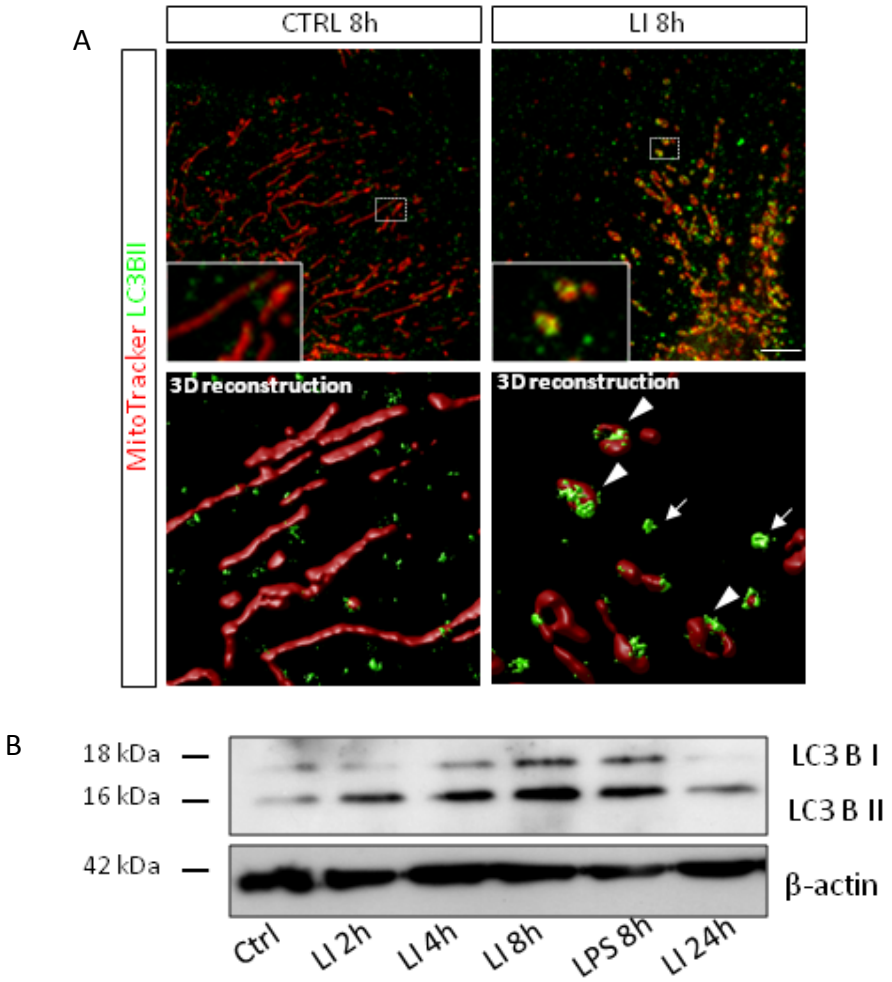


Figure 13. **Inflammation-mediated upregulation of autophagy.** ***(A)*** Representative pictures (merged channels) of cultured astrocytes incubated with MitoTracker Red and immunostained for LC3BII, a specific marker for autophagosomes. Lower panels show 3D reconstruction of selected portions demonstrating the colocalization between the two signals (arrowheads). Bar, 5 µm. ***(B)*** Time course of LC3B lipidation after inflammation analyzed by western blot. Note that even LPS alone (8 hours) is able to induce autophagy.

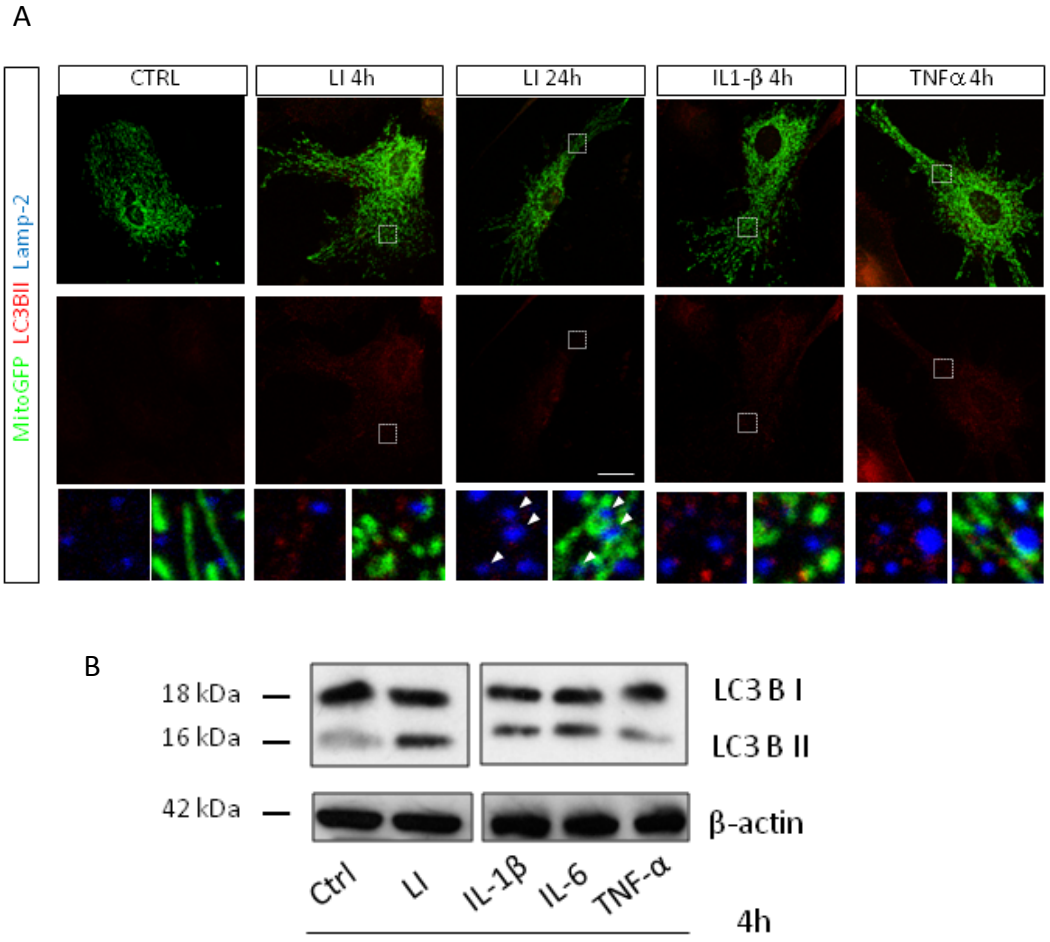


Figure 14. **Autophagic markers co-localize with fragmented mitochondria: mitochondria are fated to mitophagy during inflammation.** ***(A)*** High resolution confocal images of astrocytes expressing mito-GFP (to visualize mitochondria) treated either with LI or with different cytokines (IL-1β, TNF-α) at given time-points, and immunostained with markers for autophagy (LC3BII) and lysosomes (Lamp-2). Cytokines-induced mitochondrial rearrangement is similar to that observed upon LI treatment. Magnification of selected regions (dashed squares) is showed in the lower panels. For each condition, the colocalizations between autophagosomes and Lamp-2 (left squares) or mitochondria and Lamp-2 (right squares) are depicted. Significant colocalzation of the three signals (arrowheads) is present only 24 hours after treatment. Bar, 10 μm. ***(D)*** Western blot analysis shows that treatment of astrocytes with the upper mentioned cytokines induced a comparable induction of autophagy as LI does.

1.8. Inflammation triggers autophagy in astrocytes of cortical brain slices

While we have shown that pro-inflammatory molecules can elicit the formation of autophagosomes onto altered individual mitochondria, it is currently not known if autophagy represent a physiological mechanisms in astrocytes in response to inflammation within the native brain tissue. To address this point we performed additional experiments in acute brain slices derived from hGFAP-GFP mice, in which GFP is expressed under the control of the human GFAP promoter (Nolte, Matyash et al. 2001). In these mice, type I and II astrocytes are selectively expressing GFP, and therefore it becomes easier to reveal their finest morphology even without immunostaining (Fig. 15A-B). Acute slices were prepared from 4 to 6 weeks old mice and maintained in oxigenated artificial cerebrospinal fluid (ACSF) containing or not LPS and IFN-γ. Incubation in ACSF for 6-8 hours induced a remarkable increase in GFP expression, indicative of up-regulation of GFAP (which represents a marker of gliosis) as shown in Fig 15C. Following fixation of these slices, confocal acquisition of individual astrocytes and 3D reconstruction of the acquired z-stacks (Fig. 15D), we analyzed the degree of colocalization with the autophagic marker LC3B-II. While GFP positive astrocytes of ACSF only treated slices (CTRL) displayed minimal colocalization with LC3B-II, both type I and II astrocytes (revealed by their different morphological aspect) showed a net increase in the percentage of GFP signal colocalizing with the autophagic marker (Fig. 16A-C) These results further corroborate the previous observation obtained in primary cultures, and indicate that inflammation reliably induce autophagy in cortical astrocytes in brain slices.

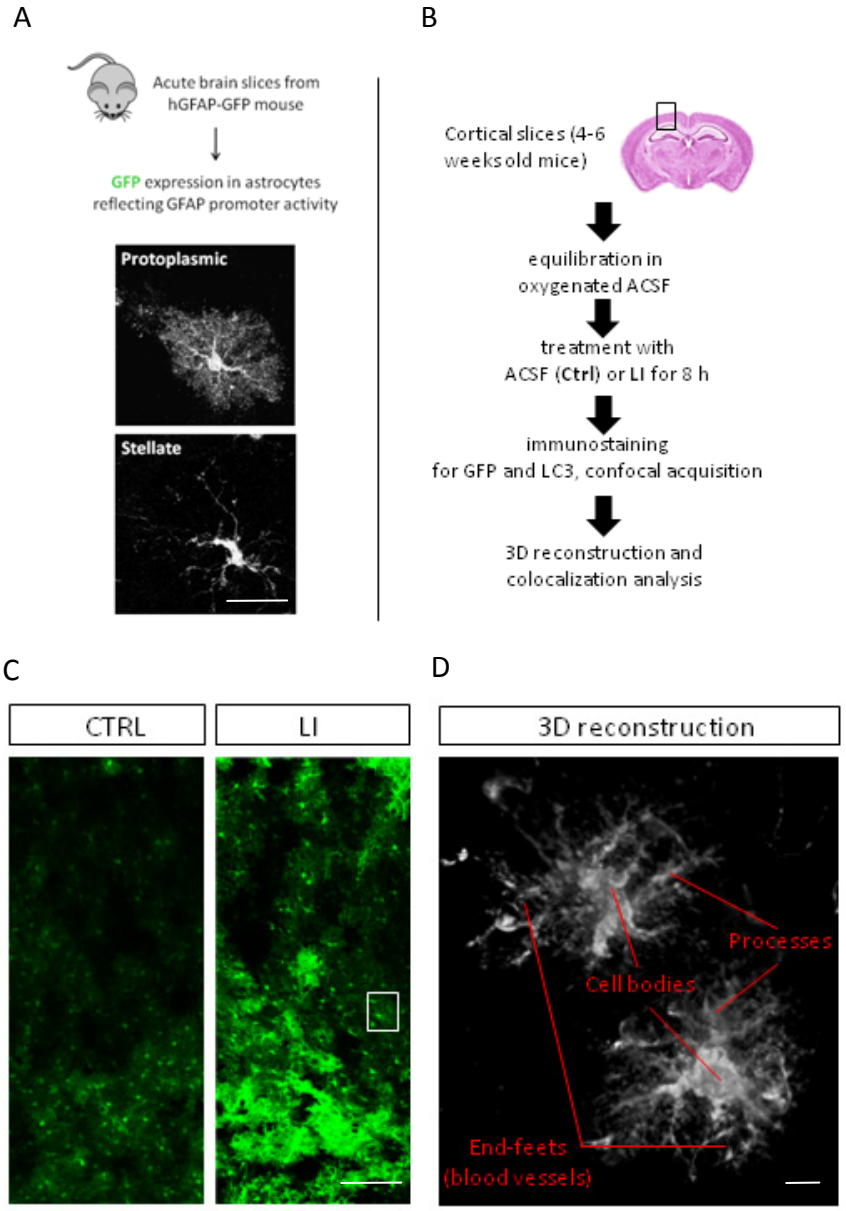


Figure 15. **Induction of inflammation in acute slices of hGFAP-GFP mice.** ***(A)*** The utilization of hGFAP-GFP mice allows visualizing the detailed morphology of the two types of astrocytes (protoplasmic and stellate) present within cortical layers. Representative confocal pictures of these two types showing the differences in branching are shown. Bar, 20 µm. ***(B)*** Schematic description of the experimental plan for indrucing inflammation in acute slices. ***(C)*** Representative confocal pictures of somatosensory cortical layers I to VI showing the increase in GFP immunoreactivity upon LI treatment. Bar, 100 µm. ***(D)*** 3D reconstruction of two selected astrocytes in ***(C)*** reveals the fine morphology of these cells. Bar, 10 µm.

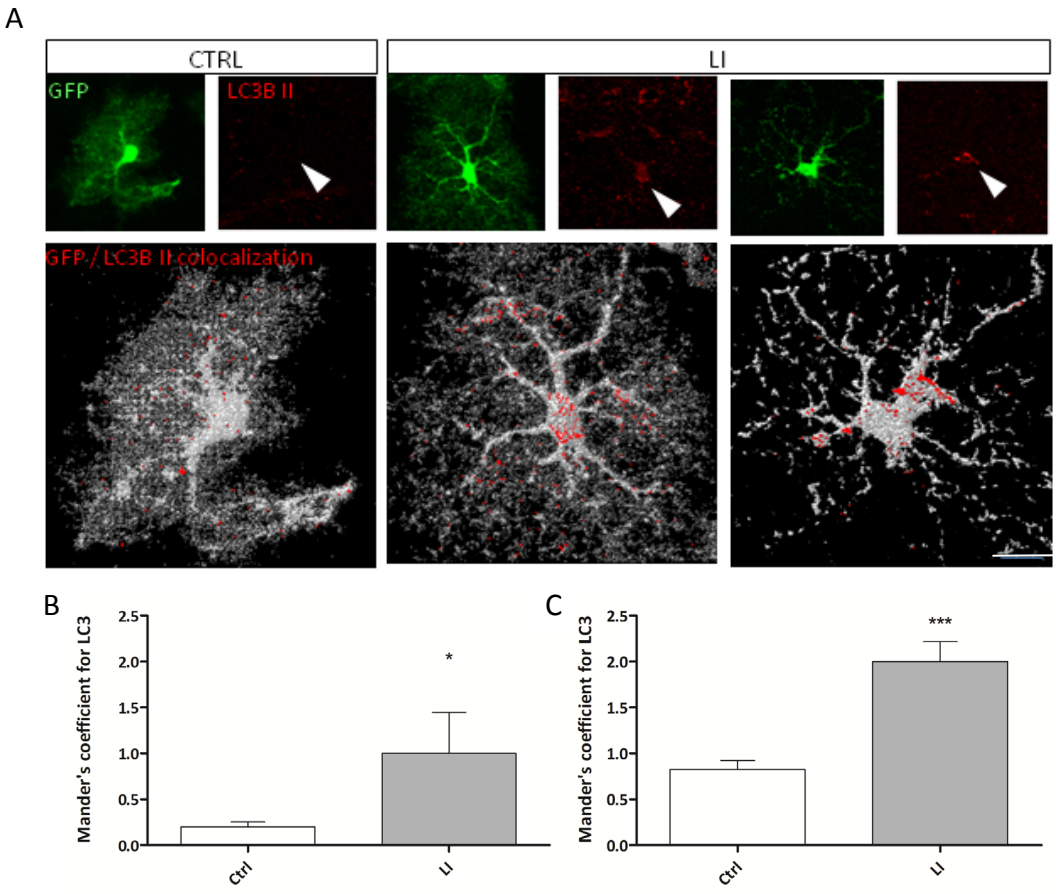


Figure 16. **Inflammation triggers autophagy in astrocytes of cortical brain slices.** ***(A)*** Representative confocal pictures of astrocytes taken from acute brain slices. After treatment, slices were fixed and immunostained for GFP and the autophagic marker LC3BII. Arrowheads point to the cell bodies in the LC3BII single channel images. Reconstruction of acquired z-stacks is shown in the lower panels to highlight the colocalization between LC3BII (red) and astrocytic area (grey). Bar, 10 μm. Colocalization analysis (Mander's coefficient) in protoplasmic ***(B)*** or stellate astrocytes ***(C)*** from Ctrl and LI-treated slices.

1.9. Abolishment of the autophagic machinery results in accumulation of hyperelongated mitochondria upon inflammation

To further understand the role of autophagy in mitochondrial quality control during inflammation, we investigated mitochondrial morphology of astrocytes hampered in their autophagic machinery for either the elongation (a step exerted by the molecule Atg7) or the conjugation (in which the molecule Atg4 plays a critical role) phases of the autophagosome formation. Fig. 17 recapitulates the steps targeted by the different strategies used in these experiments.

To interfere with Atg7, we performed experiments on astrocytes obtained from conditional knock-out mice for Atg7 ($Atg7^{fl/fl}$), a protein involved in the membrane elongation of the immature autophagosome (Komatsu, Waguri et al. 2005). Once reaching 60 to 70% of confluency, astrocytes were transduced with retroviruses encoding for either the recombinase Cre and GFP (Cre virus) or for GFP alone (control virus). 5 to 6 days after viral transduction, a significant proportion of astrocytes (more than 60%) also encoded for the reporter gene GFP, allowing to distinguish between floxed and non-floxed cells (Fig. 18A). We first confirmed the lack of Atg7 protein from Cre-transduced cultures by performing a western blot for Atg7: a clear reduction of the protein levels was detectable in floxed cultures, which indicates that gene deletion and the subsequent depletion of Atg7 occurred (Fig. 18C). In the following, we analyzed the mitochondrial network of transduced cells by immunostaining. While transduction with the control GFP-expressing virus did not perturb the course of mitochondrial rearrangement observed during inflammation (Fig. 18A-B), with mitochondria acquiring the typical rod-like shape 4 hours after inflammation and recovering by 24 hours, astrocytes transduced with the Cre-expressing virus exhibited a rather different phenotype. Cre-expressing astrocytes showed already a mild increase in mitochondrial fragmentation even in absence of inflammation, which is consistent with the fact that Atg7 deletion in mammalian cells induces the accumulation of damaged mitochondria over time due to its role in allowing mitochondrial turn-over under basal conditions (Komatsu, Waguri et al. 2005). However, following inflammation these floxed astrocytes

showed a very unusual mitochondrial network, which was characterized by the presence of hyper-elongated mitochondria (Fig.18A-B). Such a phenotype was visible at 4 hour but became mostly prominent 24 hours after LI-treatment, when the mitochondria of control cells normally returned to a tubular morphology (Fig.18A-B). To manipulate the conjugation phase of autophagosomal formation, we ectopically expressed a mCherry-tagged form of $Atg4B^{C74A}$, in which the protease Atg4B has been rendered inactive by introducing the point mutation C74A (Fujita, Hayashi-Nishino et al. 2008). By introducing this construct, autophagy becomes inhibited since the Atg4B protease is responsible for the cleavage of LC3B-I into LC3B-II, an essential step in the proper closure and maturation of the autophagosome (Fujita, Hayashi-Nishino et al. 2008). As shown in Fig. 19, following the transfection of low amounts of $Atg4B^{C74A}$-encoding plasmid, reporter positive (mCherry) control astrocytes showed the typical tubular mitochondrial network, indicating that this mutant protein does not cause significant toxicity to these cells under basal conditions. Interestingly, inflammation of astrocytes lacking autophagy again resulted in the characteristic appearance of hyperelongated mitochondria, as observed following deletion of Atg7. This phenotype was even more dramatic 24 hours after induction of inflammation, with transfected cells showing a complete disruption of the typical mitochondrial network, which was instead replaced by clusters of hyperelongated mitochondria.

Therefore, impairment of the autophagic machinery achieved by 2 indipendent experimental approaches resulted in a very similar phenotype when inflammation was induced: astrocytes did not respond by rearranging their mitochondrial network as we observed in presence of functional autophagy. Rather, mitochondria - which we observed to be normally fated to mitophagy and targeted to lysosomes at later stages – were spared from fragmentation through hyper-elongation, a mechanism which has recently been described in cell lines during starvation-induced cellular stress (Gomes, Di Benedetto et al. 2011; Gomes and Scorrano 2011; Rambold, Kostelecky et al. 2011). Here, however, this second pathway of mitochondrial rearrangement became prominent only once autophagy (and therefore mitophagy)

was impaired, providing a new mechanism for the maintenance of damaged mitochondrial once their turn-over becomes impaired.

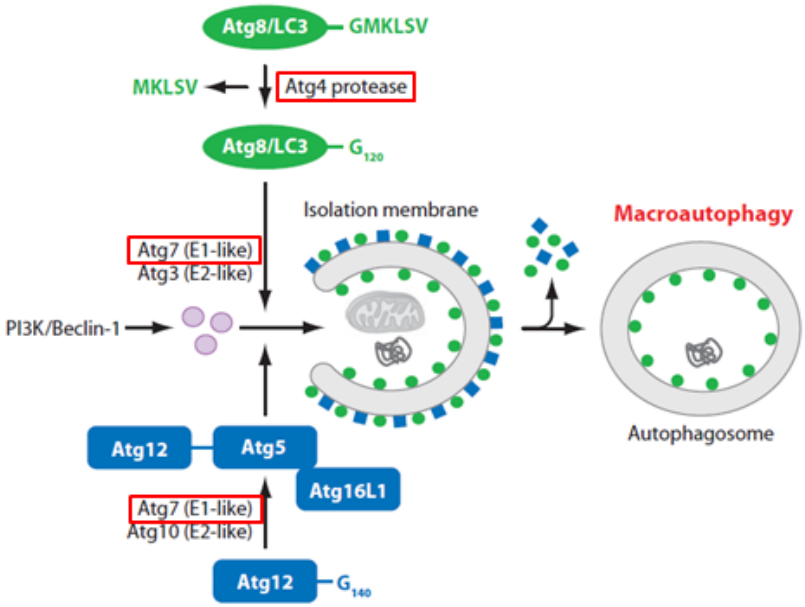


Figure 17. **The autophagic machinery** Scheme depicting the steps and key proteins required for the generation of an autophagosome. Atg4 and Atg7 (red rectangles) represent the critical protein manipulated in this study to block autophagy.

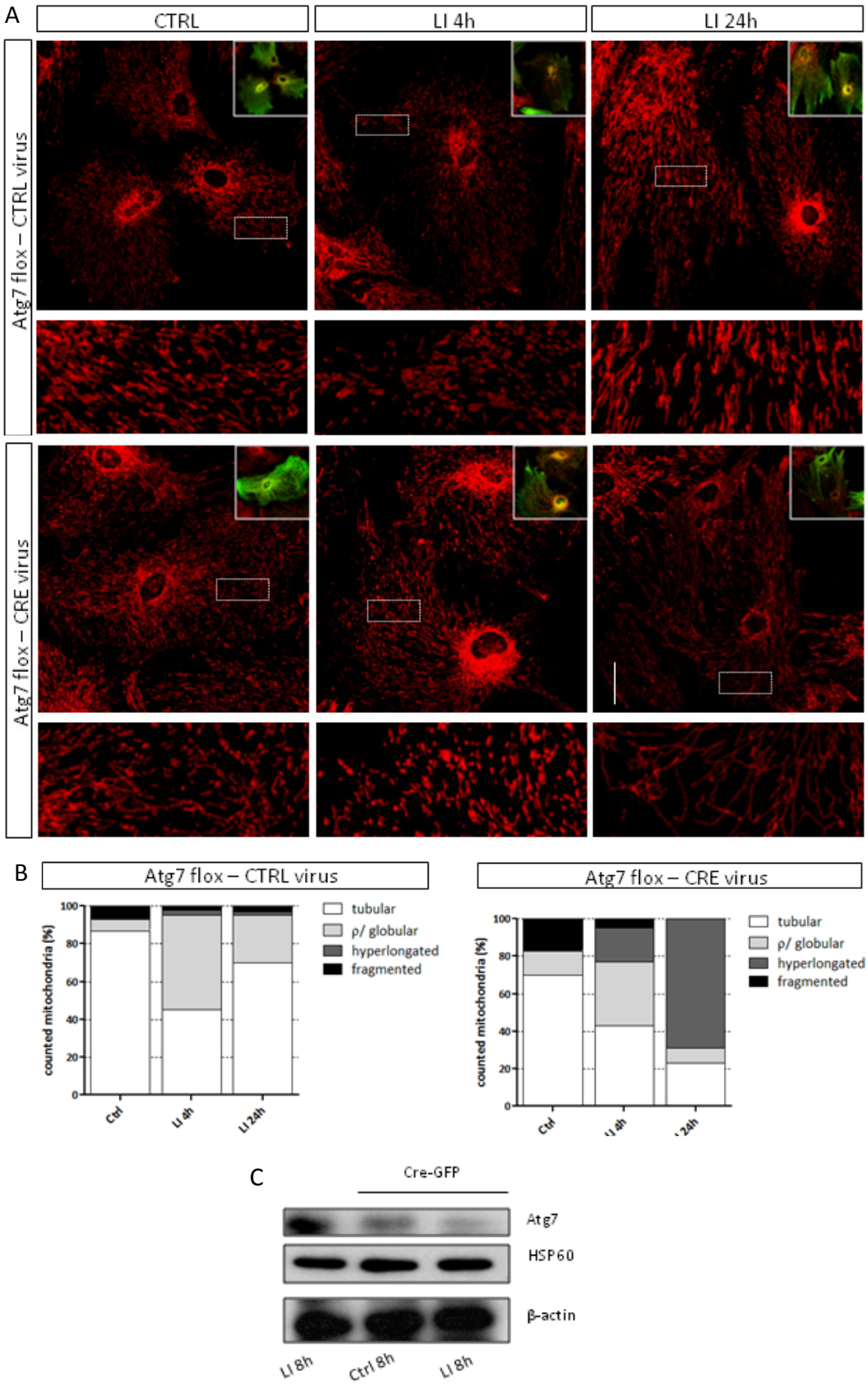

A
CTRL
LI 4h
LI 24h
Atg7 flox – CTRL virus
Atg7 flox – CRE virus
B
Atg7 flox – CTRL virus
Atg7 flox – CRE virus
counted mitochondria (%)
tubular
ρ/ globular
hyperlongated
fragmented
Ctrl
LI 4h
LI 24h
C
Cre-GFP
Atg7
HSP60
β-actin
LI 8h
Ctrl 8h
LI 8h

Figure 18. **Inhibition of autophagy via conditional deletion of Atg7 causes hyperelongation of mitochondria following inflammation** ***(A)*** Representative confocal pictures of astrocytes derived from Atg7 flox mice and transduced with a GFP (control) or CRE-GFP encoding virus. Astrocytes were subjected to inflammation and the morphology of their mitochondria (immunostained for Tom20) compared with untreated astrocytes. Inset show merged channels (GFP = green, Tom20 = red). Lower panels depict magnifications of the indicated white rectangles. While GFP-transduced astrocytes return to a normal mitochondrial network 24 hours post-inflammation, CRE expressing astrocytes maintain a hyperelongated network. Bar, 10 μm. ***(B)*** Quantification of mitochondrial morphology following viral transduction as illustrated in A. ***(C)*** Efficiency of the virus-mediated knock-out for Atg7 evaluated by western blot.

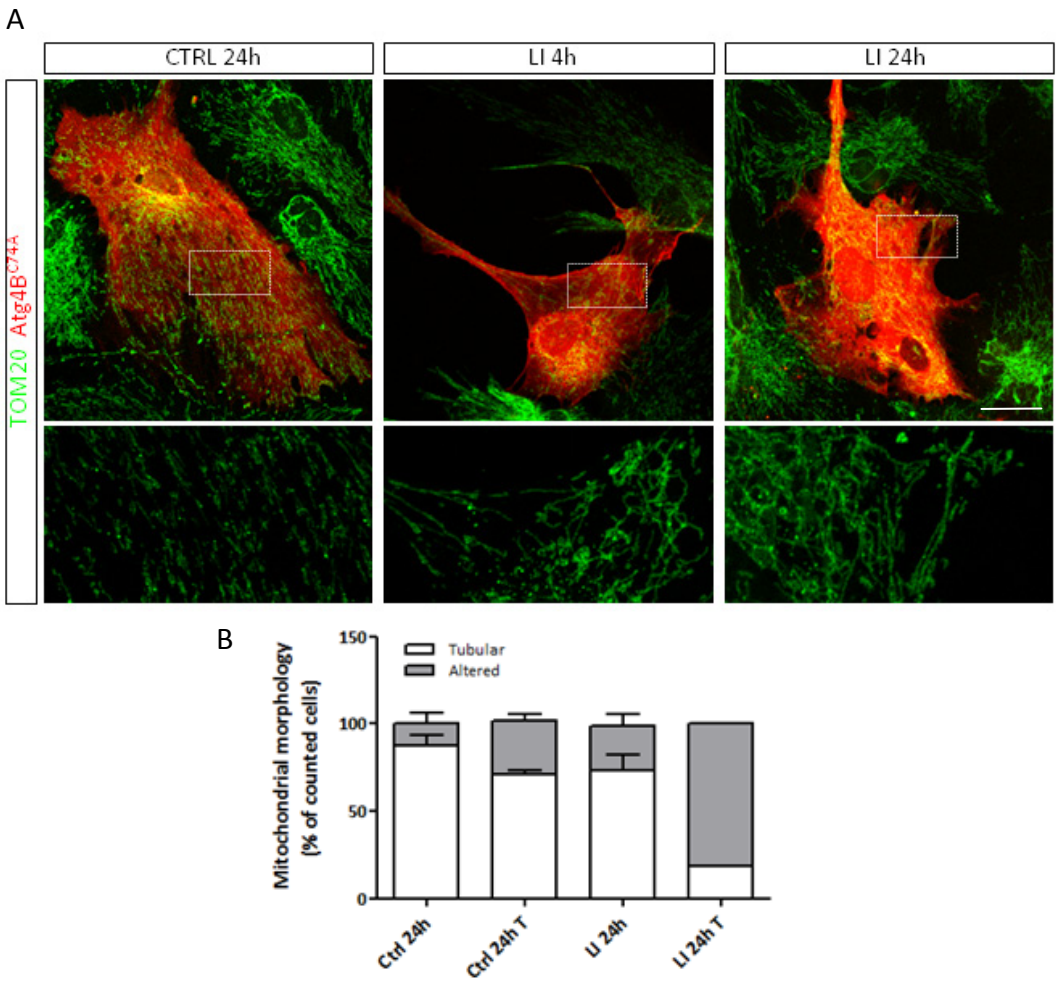


Figure 19. **Inhibition of autophagy by impairing Atg4 produces hyperelongated mitochondria following inflammation** ***(A)*** Confocal pictures of astrocytes transfected with a plasmid encoding DsRed and a dominat negative mutated isoform of Atg4 (Atg4^{C74A}), which blocks the autophagic cascade. Astrocyes were fixed and immunostained for Tom20 to visualize mitochondria. Starting from 4 hours post-inflammation, mitochondria appear hyperelongated. Bar, 10 μm. ***(B)*** Quantification of mitochondrial morphology following trasfection of a control plasmid or the Atg4^{C74A} plasmid shown in A (indicated with the letter T). The graph demonstrates that hyperelongated mitochondria persist until 24 hours, when astrocytes transfected with the control plasmid mainly rescue their mitochondrial network.

1.10. Inflamed astrocytes mediate neuronal degeneration

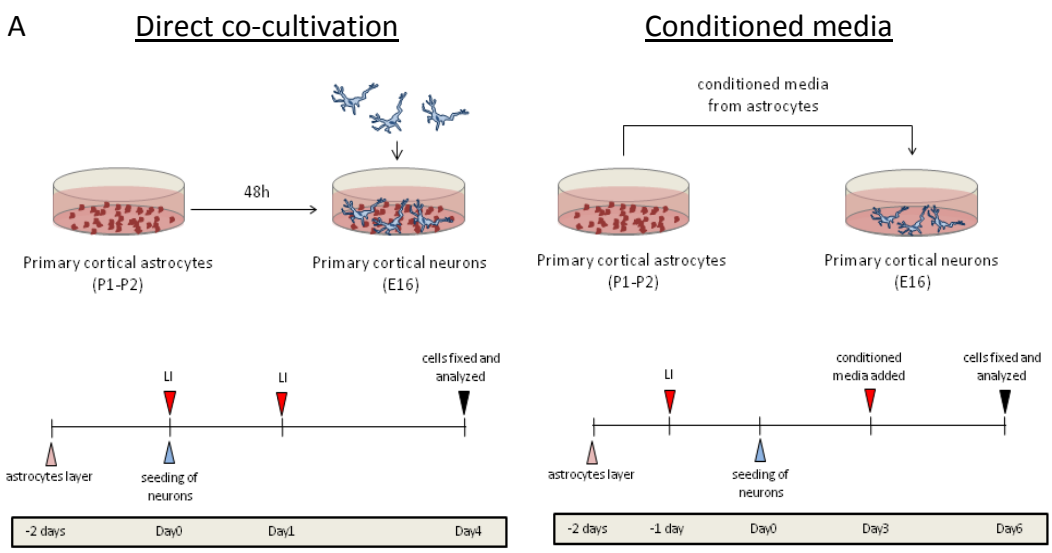
It has been recently demonstrated that activation of microglia is a critical step in inflammation-mediated neurotoxicity (Block ML et al 2007). We then investigated if astrocytes, like microglia, could contribute to this neurotoxicity during the inflammatory response. To this aim, we established co-cultures and conditioned media experimental models to dissect out a possible contribution of astrocytes in preserving neuronal viability or promoting neurodegeneration. The main difference between these two models is that while in the co-culture system the direct contact between neurons and astrocytes mimics a more physiological situation, the growth of neurons in medium conditioned from separated cultures of astrocytes allows to selectively study the effects mediated by secreted astrocytic factors which are released into the medium (Fig. 20A).

First of all, we tested whether the inflammatory molecule that we used in the course of this study could have an impact on the viability of pure neuronal cultures. Neurons were treated with either LI or IL-1β, IL-6 and TNF-α for up to three days, after which we quantified the induction of active-caspase3, a well used marker for assessing apoptosis (Bossy-Wetzel and Green 1999). Only LI treatment revealed to produce no detectable effects on neuronal viability, while all the others citokines produced an increase in neuronal cell death indicating that they are toxic to neurons as previously reported (Cunningham, Murray et al. 1996; Friedlander, Gagliardini et al. 1996; Carlson, Wieggel et al. 1999). Based on these results, we decided to use LI for testing the effect of inflamed astrocytes on neuronal viability both in conditioned media experiments and in co-cultures experiments.

Fig. 20A summarizes the paradigms used for the two experimental approaches. In conditioned media experiments, astrocytes were treated with LI for either 8h or 24h. This medium was then transferred into neuronal cultures and the amount of apoptosis was quantified 72 hours later. Under these conditions, we could detect comparable numbers of activated caspase-3 positive neurons in cultures receiving conditioned media from either untreated or inflamed astrocytes (Fig. 20C). However, when neurons were grown in direct contact with astrocytes (co-cultures), the pre-treatment or co-treatment of these astrocytes with LI caused a dramatic increase in the number

of activated caspase-3 neurons (Fig. 20B), independently of whether inflammation was induced one day before (Day 0 = D0) or immediately after (Day 1 = D1) plating the neurons onto the astrocytic layer (D0, fold increase vs control = 1.580±0.116, $P< 0.01$; D1, fold increase vs control = 1.833±0.154, $P< 0.001$).

Moreover, experiments conducted in co-cultures revealed that also neuronal morphology was affected by astrocytic inflammation: by staining co-cultures for the neuronal marker MAP-2, which discloses the morphology of cells, neurite outgrowth appeared halted upon induction of inflammation in comparison with neurons grown onto untreated astrocytes (Fig. 20E). These results suggest that the functional changes induced by inflammation in astrocytes, and possibly the alterations in their energetic status or in their release of potential short-range toxic mediators such as NO or mitochondrial ROS, can impact the survival of neurons and therefore contribute to their degeneration during the inflammatory reaction.


A
Direct co-cultivation
Conditioned media
48h
Primary cortical astrocytes
(P1-P2)
Primary cortical neurons
(E16)
conditioned media
from astrocytes
Primary cortical astrocytes
(P1-P2)
Primary cortical neurons
(E16)
LI
LI
cells fixed and
analyzed
astrocytes layer
seeding of
neurons
-2 days
Day0
Day1
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Day0
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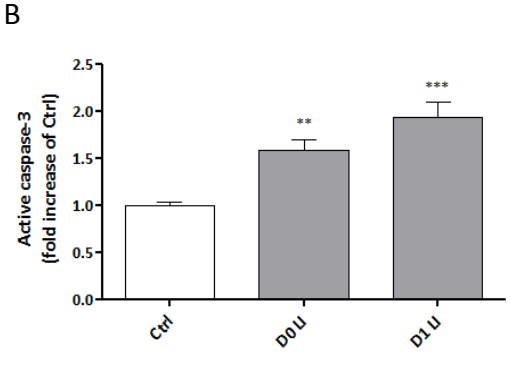
Day6


B
Active caspase-3
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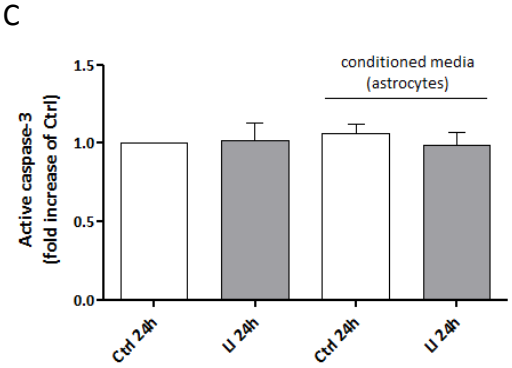
C
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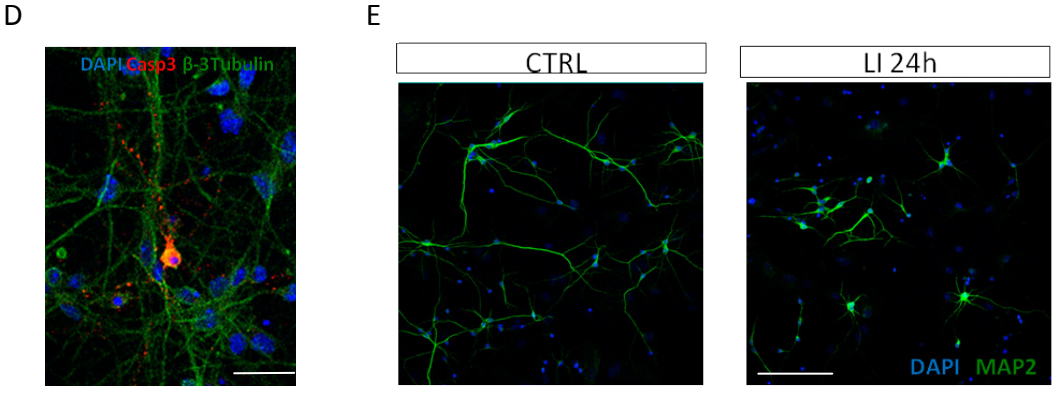
D
DAPI Casp3 β-3Tubulin
E
CTRL
LI 24h
DAPI MAP2

Figure 20. **Inflamed astrocytes mediate degeneration of co-cultured neurons** ***(A)*** Schemes depicting the two methods used for assessing the putative contribution of inflamed astrocytes to neurodegeneration. The left scheme illustrate the co-coltures of cortical astrocytes and neurons; on the right, the method used for testing the contribution of conditioned media deriving from inflamed astrocytes. The experimental plan is illustrated for both methods on the bottom. ***(B, C)*** Quantification of Caspase-3 active (Casp3) positive neurons (identified through immunostaing for neuronal marker beta-3 tubulin) obtained following co-cultures **(B)** or the conditioned media **(C)**. The amount of neuronal death following direct treatment of neurons with LI is shown in C. ***(D)*** Representative picture showing a Casp3 positive neuron undergoing degeneration. Bar, 20 μm. ***(E)*** Confocal pictures of beta-3 tubulin positive neurons in co-cultures with control or inflamed astrocytes. Neurons display evident reduction of their neuritic length and a less prominent morphological development. Bar, 50 μm.

2. Discussion

The major finding of the present study is that cortical astrocytes subjected to pro-inflammatory stimuli undergo a rapid rearrangement of their mitochondrial network. In turn, this mitochondrial rearrangement initiates the autophagic response aimed at degrading damaged mitochondria. Strikingly, astrocytes do not die upon inflammation, consistent with the fact that their mitochondrial pool is maintained and gradually returns toward a normal tubular network by 24 hours post-inflammation.

The original observation that the exogenous application of individual inflammatory molecules (such as LPS, IFN-γ, TNF-α, IL-1β and IL-6) is sufficient to induce mitochondrial remodeling, strongly suggests that astrocytes immediately reorganize their metabolism and cellular architecture once cytokines become released (for example by microglia) in the local environment during neuroinflammation. This mitochondrial rearrangement, which appears to terminate within 24 hours from the insult in vitro, may be part of an intracellular metabolic pathway specific to signal an ongoing inflammatory process in astrocytes. Eventually, astrocytes could then employ mitochondria to continuosly probe the extent of inflammation and locally secreted cytokines. As astrocytes not only provide a trophic support for neurons, but also influence their synaptic transmission and regulate as well the flux of blood by controlling the diameter of local capillaries, their capability to quickly monitor the local amount of pro-inflammatory molecules via mitochondria could allow them to co-regulate synaptic and neuronal viability from the very beginning of an inflammatory process. On the other side, by means of time-lapse single-cell imaging, we demonstrated that mitochondria rapidly reduce their length, motility and progressively cluster toward the perinuclear region of the cells in vitro. Although the morphology of astrocytes in cultures clearly differs from their native morphology in the brain, where they show profuse branching and contacting of neuronal synapses and cell bodies, these observations indicate that once altered upon inflammation, the mitochondria located in whichever process of the cell will tend to remain segregated in that specific process, thus avoiding the rest of the mitochondrial network to become affected by this phenomenon. This speculation is further supported by experiments in which we analyzed the fusion proficiency of these organelles

following inflammation. As the mitochondria of inflamed astrocytes severely reduce their capability to fuse with other mitochondria, it is conceivable to imagine that astrocytes also initiate “safe mechanisms” in order to isolate the perturbed sub-cellular region adjacent to the inflamed tissue and, at the same time, to maintain functional the remaining pool of mitochondria. With a similar mechanism, Ca^{++} transients in astrocytes follow a sub-cellular regionalized pattern of appearance. Recent works demonstrate that, within the tissue, astrocytes respond to synaptic activity of neurons with local calcium transient confined to the region of the astrocytic process closer to the activated synapse (Bernardinelli, Salmon et al. 2011; Di Castro, Chuquet et al. 2011). Since Ca^{++} elevations represent the principal way of signaling and communication of astrocytes, it would be interesting to investigate if these transients are among the very first intracellular events associated with the mitochondrial rearrangement observed during inflammation.

It is interesting to note that the mitochondrial rearrangement induced by inflammation appears to be dependent on the up-regulation of iNOS, and therefore from NO production. The expression of iNOS is indeed widely used as a marker to assess if and when inflammation is taking place (Bal-Price and Brown 2001; Borutaite, Hope et al. 2006). Furthermore, NO represents a classical gaseous second messenger which can control numerous intracellular signaling pathways (Almeida, Almeida et al. 2001; Almeida and Branco 2001; Bal-Price and Brown 2001; Bolanos, Garcia-Nogales et al. 2001; Bossy-Wetzel and Lipton 2003; Barsoum, Yuan et al. 2006), but has as well the capacity to diffuse extracellularly thereby acting in a paracrine fashion on neighboring cells, such as other glial cells and neurons, or on blood vessels, in which NO mediates vasodilatation (Gordon, Jain et al. 2002). The observation that a pharmacological inhibitor of iNOS, such as L-NAME, can prevent astrocytes from altering their mitochondrial dynamics, suggests that (i) NO is likely to be upstream of this rearrangement and (ii) the same second messenger used to communicate with other local cells is involved in cell-autonomous mitochondrial rearrangements. The effect observed here on mitochondrial morphology is not totally unexpected. Indeed, a role of exogenously administered NO (via NO donors) and cyclic GMP (as it represents one of the intracellular mediators of NO) was previously

reported to induce mitochondrial fission in cultures of cortical neurons and in myogenic precursors, in which this NO-mediated effect on mitochondria was linked to the action of the GTPase Drp1 (Barsoum, Yuan et al. 2006; Yuan, Gerencser et al. 2007). As the use of Drp1 mutants prevented fission during NO application, whereas overexpression of Drp1 elicited fission even independently of NO (Barsoum, Yuan et al. 2006), the data provided by these papers and translated in a context of inflammation would suggest here that the rapid production of NO followed by cyclic GMP could ultimately determine an unbalance of the physiological fusion-to-fission ratio and therefore promote mitochondrial fragmentation or the appearance of rod-like mitochondria. This scenario would explain why, following application of an inhibitor of iNOS (L-NAME), mitochondrial fragmentation is prevented during inflammation.

Clearance of permanently damaged mitochondria via mitophagy

The second most important observation of this study is that astrocytes undergo autophagy after induction of inflammation, both in cultures and in brain slices. Contrary to many reports in which a massive autophagy becomes detectable under stress conditions (Mizushima, Levine et al. 2008; Criollo, Senovilla et al. 2010; Mazure and Pouyssegur 2010), starvation (Schwarzer, Dames et al. 2006; Jia and Levine 2007; He and Klionsky 2009) or during cellular degeneration (Debnath, Baehrecke et al. 2005; Adi-Harel, Erlich et al. 2010; Danial, Gimenez-Cassina et al. 2010; Dorn 2010), here this process can be reliably detected within 2-4 hours post-inflammation, peaks around 8 hours but gradually returns to baseline levels after 24 hours. This unique pattern of activation and deactivation triggered for further investigation and led to the question whether autophagy in astrocytes was linked to the time-matched mitochondrial rearrangement observed after treatment with pro-inflammatory molecules. Surprisingly, many of the fragmented mitochondria observed in these conditions were indeed targeted by nascent autophagosomes. In addition, at later time-points, when the majority of the mitochondrial network was re-established, those mitochondria which maintained an altered morphology were also

immunoreactive for lysosomal markers, suggesting their degradation via the mitophagic process.

In the last years, many papers have reported mitophagy as a common mechanism used by cells to get rid of damaged mitochondria, a conclusion often supported by electron-microscopic studies in which clearly altered mitochondria appeared contained within autophagosomal structures (Klionsky, Abeliovich et al. 2008). In many of these studies, however, such an extent of autophagy has been obtained following extensive detrimental treatments of cell lines which do not allow for the cells themselves to recover after the initial insult, with the problem that any subsequent interpretation regarding the functional role of mitophagy becomes more complicated if not impossible. Here, inflammation of astrocytes does not cause their death. This allowed us to monitor the autophagic response across time until its complete resolution, both in fixed cells and in live imaging. In addition, blockage of autophagy through removal of specific key proteins, such as Atg4 or Atg7, revealed that astrocytes deviate from a more physiological course of mitochondrial clearance (mitophagy) toward the establishment of a hyperfused mitochondrial network when mitophagy is not available. This effect is reminiscent of what has been observed in cell lines or mouse embryonic fibroblasts when cells were subjected to stressful conditions such as nutrients deprivation (Rambold, Kostelecky et al. 2011; Rambold, Kostelecky et al. 2011) or proapoptotic stimuli such as UV irradiation (Tondera, Grandemange et al. 2009). In these cases, cells reacted with an hyperelongation/fusion of mitochondria with the aim of either maintaining their ATP production (Tondera, Grandemange et al. 2009; Rambold, Kostelecky et al. 2011) or sparing them from mitophagy (Tondera, Grandemange et al. 2009), therefore sustaining cell viability. In the case of astrocytes and inflammation, however, it was the genetic abrogation of the autophagic response which initiated the hyperfusion of mitochondria, as if the cells decided to adopt this modality to avoid accumulating damaged mitochondria. It is conceivable that, rather than having clusters of mitochondria potentially harmful for the astrocyte (as they produce high amounts of ROS), their reorganization into an hyperfused network may help, to some extent, (i) in maintaining mitochondrial functionality and (ii) in reducing the risk to generate

loci of high radicals production. Future studies will be required to assess ROS production and mitochondrial potential in hyperelongated mitochondria of autophagy-deficient astrocytes following inflammation.

Potential physiological relevance of the astrocytic response to inflammation for their cross-talk with neurons

To date, all described functions exerted by astrocytes are ultimately aimed to modulate the excitability, metabolism and homeostasis of neurons. Here, we sought to examine, after inflammation, which potential effects astrocytes could determine on neuronal survival in culture. A plethora of molecules released by astrocytes are known to regulate neuronal physiology (Volterra and Meldolesi 2005; Barres 2008), some of which possess a remarkably reduced half-life, such as ROS and RNS (Hirrlinger and Dringen 2010). Therefore, we established two experimental methods to address the issue of how astrocytes could impact neuronal viability: in the first one, we co-cultured inflamed astrocytes with cortical neurons; alternatively, we studied the influence of conditioned media derived from inflamed astrocytes on separate pure neuronal cultures. Conspicuously, we observed an increase in neuronal apoptosis upon inflammation only when the two cell types were co-cultured, suggesting that astrocytes release compounds toxic for neurons under this condition, which action requires a direct contact with neurons. This effect was not observed in conditioned media experiments, strengthening the idea that neither peptides nor classical gliotransmitters – which function can instead be reliably studied in this kind of experiments– play a role in the neuronal apoptosis observed in co-cultures.

An important pre-requisite for reaching appropriate conclusions from these experiments concerns the possibility that neurons directly sense, and in turn react to, the applied pro-inflammatory molecules used in this study. Indeed, if neurons would respond to these by undergoing apoptosis in a cell-autonomous manner, the subsequent observed effect would be more difficult to be dissected. To this aim, we first tested the reactivity of neurons to cytokines and to LPS. Indeed, we observed a direct effect of TNF-α, IL-1β and IL-6 on neuronal viability (data not shown), but no

clear effect was induced by direct administration of LPS and IFN-γ (at the used concentrations). Based on these observations, we decided to perform co-cultures and conditioned media experiments only with this last combination of molecules, thus restricting the cause of any following observed effect to the action of astrocytes. Yet, whether such a role of inflamed astrocytes in mediating neuronal death represents the consequence of a rather high concentration of LPS and IFN-γ used in vitro is not clear. Indeed, this may not be case in vivo, where a more physiological inflammation process taking place is characterized by the action of other cell types than astrocytes, playing a role by both secreting and removing pro- and anti-inflammatory mediators (Farina, Aloisi et al. 2007; Barres 2008). Therefore, one possibility is that the model here proposed following in vitro co-culturing of astrocytes and neurons closely resembles a chronic inflammation, in which a massive and prolonged secretion of pro-inflammatory mediators takes place. Again, future studies will be required to understand (i) whether the pro-neurodegenerative role of inflamed astrocytes depends to some extent on the excessive mitochondrial release of radicals and (ii) if astrocytes, rather than promote, buffer local inflammatory molecules by undergoing autophagy and thus protecting nearby neurons from a similar detrimental insult.

MATERIALS AND METHODS

1. Mice

Time-pregnant C57B6 mice, C57B6 pups and hGFAP-GFP mice (provided by Magdalena Goetz, LMU Munchen, Germany; ref) were used in this study. All animal procedures were performed in accordance with the Italian, Bavarian and European Union guidelines and were approved by out institutional animal care and utilization committee.

2. Viral Vectors

The murine Moloney leukemia virus (MoMulV)- based vector CAG-GFP (Zhao et al, 2006) and CAG-GFP IRES-Cre were provided by Matteo Bergami (LMU Munchen, Germany). The final measured titer was about $5x10^7$ viral particles/ml.

3. Cell cultures

Astrocytes. Primary cultures of cortical astrocytes were prepared from postnatal day 1-2 wild-type and $Atg7^{fl/fl}$ mice (provided by T. Misgeld, Technische Universität, Munchen, Germany) as previously described (McCarthy and de Vellis 1980). Briefly, superficial cortical layers were dissected out in chilled 0.1M phosphate-buffer solution (PBS), mechanically triturated in Dulbecco's Modified Eagle Medium F12 (DMEM-F12, Lonza), filtered with a 75 μm cell strainer (Millipore), plated in plastic flasks (Corning), and mantained in DMEM-F12 with 10% fetal bovine serum (GIBCO) at 37°C in 5% CO_2. Flasks were shaked every 3 days and medium replaced until confluency was reached (about 2-3 weeks after plating). For experiments conducted in poli-D-lysine-coated coverslips, astrocytes were trypsinized and 100,000 cells/coverslip (15 mm, 1) were used for subsequent experiments.

Neurons. Primary cultures of cortical neurons were prepared from embryonic mice at embryonic day E15.5 as follows. Superficial cortical layers were dissected out in

chilled Hank's Buffer (HBSS, Invitrogen), trypsinized for 20 min at at 37 °C in dissociation medium (200 U of papain, Sigma, in DMEM Glutamax, Invitrogen) , and thereafter washed three times in DMEM Glutamax. Cells were then dispersed with a 2mL pipette and cultivated in DMEM Glutamax onto poli-D-lysine-coated coverslips for 3h. After this time, medium was changed into growth medium, composed of Neurobasal (Invitrogen)+ 1% of B27 supplement (Invitrogen)+ 0.05 mM Glutamine (Invitrogen).

Co-cultures of astrocytes and neurons. Primary cultures of cortical neurons were prepared as reported above, with the following modifications. After dissociation of the cortex with papain, cells (45,000/ml) were resuspended directly in Neurobasal+B27 and plated onto a layer of astrocytes previously equilibrated 24h in Neurobasal+B27.

Conditioned media. Primary cultures of cortical neurons and astrocytes were prepared separately as reported above. Astrocytes cultures were first equilibrated 24h in Neurobasal+B27, then treated for the given time-points. Medium derived from either untreated or treated astrocytes was then transferred to the neurons.

4. Western blot

Proteins were separated by SDS-PAGE and transferred to PVDF membranes using standard procedures. After blocking unspecific sites, the membranes were incubated overnight at 4°C with mouse α-beta actin (Sigma), rabbit α-LC3 (Sigma), rabbit α-iNOS (Abcam), rabbit α-Atg7 (Abcam), goat α-HSP60 (Santa Cruz) primary antibodies. Detection was performed after 60 min incubation with secondary antibodies conjugated to horseradish peroxidase (Promega) and subsequent conversion with a chemiluminescent substrate (GE-Healthcare).

5. Immunocytochemistry and Immunohistochemistry

Coverslips. Cells were fixed with 4% PFA (Sigma) in PBS, permeabilized for 5 min in 0.1% Triton X-100 (Sigma) in PBS and incubated overnight in 3% BSA (Sigma) in PBS containing the following primary antibodies: chicken or mouse anti-GFAP

1:1000 (Millipore), rabbit anti-LC3BII 1:300 (Cell Signaling), rat anti-Lamp2 (Abcam), rabbit anti-TOM20 1:1000 (Santa Cruz), chicken anti-GFP (Aves Laboratory) mouse anti-β3-tubulin 1:1000 (Millipore), rabbit anti-activated caspase-3 1:300 (Cell Signaling), rabbit anti-MAP2 1:1000 (Millipore), mouse anti-SMI132 1:500 (Millipore). Cells were then incubated for 2 h at room temperature with secondary antibodies conjugated with FITC, Cy3, Cy5 (Chemicon), Alexa 488, 546, and 647 (Invitrogen), mounted in Aqua Poly/Mount (Polysciences, Inc.), and analyzed by confocal microscopy.

Slices. Slices were permeabilized for 10 min in 0.5% Triton X-100 (Sigma) in PBS and incubated overnight in 3% BSA (Sigma) in PBS containing primary antibodies chicken or mouse anti-GFAP 1:1000 (Millipore), rabbit anti-LC3BII 1:300 (Cell Signaling), chicken anti-GFP (Aves Laboratory). Slices were incubated for 2 h at room temperature with secondary antibodies conjugated with FITC, Cy3, Cy5 (Chemicon), Alexa 488, 546, and 647 (Invitrogen), mounted in Aqua Poly/Mount (Polysciences, Inc.), and analyzed by confocal microscopy. Immunoreactivity was evaluated using a confocal laser-scanning microscope (LSM710; Zeiss Laboratories) equipped with 405, 488, 561 and 633nm laser lines and 10x, 25x, 40x, and 63x objectives (Zeiss).

6. Time-lapse video-imaging

Cortical astrocytes grown on glass coverslips were transfected 48h before imaging. Time-lapse imaging was conducted using a Zeiss Observer z1 equipped with a Yokogawa CSU CCD camera and a spinning disc unit. The lasers used had excitation wavelenght at 488 and 540 nm. During acquisition, the laser beam and exposure times were kept as lowest as possible to reduce photo-toxicity. Images were acquired using a 63X-1.3NA water immersion objective. Typical experiments were conducted for 8-12h, in which z-stack series were acquired every 3-4 min. Video images were analyzed with ImageJ (NIH). For mitochondrial motility experiments, images were acquired for a total duration of 10 min spaced by 30 sec each, in order to track individual mitochondria.

7. Cytofluorimetric evaluation of apoptosis and necrosis.

Apoptotic and necrotic events in astrocytes upon inflammation were determined by flow cytometry (Guava EasyCyte Mini, Guava Technologies,Hayward, CA), using the Guava Nexin reagent (Guava Technologies,Hayward, CA), according to the instruction provided by the manufacturer. Guava Nexin reagent contains annexin V-phycoerythrin (PE), that detects the residues of phosphatidylserine on the external membrane of apoptotic cells, and the cell impermeant dye 7-amino-actinomycin D (7-AAD), to discriminate dead ones.

Briefly, astrocytes treated with LI at different time-points were trypsinyzed and resuspended in equal amounts of PBS and Guava Nexin Reagent. Cells were allowed to stain 20 min at room temperature in the dark before measurements were taken.

8. ATP assay

Cellular steady state ATP levels were measured using the luciferase-based ATP Bioluminescence assay kit HS II (Roche Applied Science), according to the manufacturer's instructions. Astrocytes were treated for the given time-points and, where indicated, medium was replaced with a 3mM glucose medium (low glucose) 24h before harvesting the cells. Bioluminescence, indicative of the ATP content, was measured using a LB96V luminometer (Berthold Technologies) and normalized to total protein levels.

9. Nitric oxide production

Intracellular NO production was measured using DAF-FM diacetate (Invitrogen), according to the protocol provided by the manufacturer. DAF-FM is a diaminofluorescein derivative, specific for the detection of NO molecules: DAF-FM diacetate is metabolized by the cellular esterases, and its fluorescence quantum yield proportionally correlates to the amount of NO present inside the cells. Fluorescence intensity was measured using a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer) (λ_{exc} = 485 nm and λ_{em} =535 nm).

10. Slice preparation.

hGFAP-TVA male mice (4-6 week old) were anesthetized with CO^2 and the brain was quickly removed into a chilled artificial cerebrospinal fluid (ASCF) saturated with 95% O_2 and 5%CO_2 (composition in mM: 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 25 $NaHCO_3$ and 25 D-glucose; pH 7.4). Coronal brain slices containing the somatosensory cortex (250μm thick) were prepared by using a vibro-slicer (Leica) and maintained at 28°C for 1h after cutting, followed by additional 1h at room temperature. Slices were then transferred into a home-made incubation chamber and incubated for 8h into ACSF or ACSF added of LPS (1μm/ml).

11. Quantitative analysis

Colocalization analysis. Immunoreactivity to LC3 was visualized by confocal microscopy using a 63x objective, with 2x digital magnification. Z-series stacks were acquired with an interval of 0.3 μm between focal planes and processed by deconvolution. Colocalization between different signals was evaluated by calculating the Mander's coefficient with the ImageJ PlugIn JacoP (NIH).

Mitochondrial motility and speed analysis. Mitochondria (n≥50 per condition) were tracked off-line by using the ImageJ PlugIn MTrackJ, and their motility was defined by the average value of D2P parameter, that indicates the distance travelled by mitochondria between consecutive frames. Mitochondrial motility was classified in three different groups, using as a threshold value the median length of an astrocyte mitochondrion in the shorter axis, that was found to be 0.3 μm. Mitochondria were classified as static, if D2P< 0.3 μm; moving, if 0.3 μm <D2P< 1 μm; highly moving, if D2P> 1 μm. Average speed was calculated by using MTrackJ.

12. Statistical analysis

The results are presented as means ± SEM from three different experiments, and the statistical significance was determined using the unpaired Student's *t*-test.

Alternatively, data were analyzed using multiple comparison one-way ANOVA followed by Dunnett's post hoc test.

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