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The Interaction Between NCX3 and AKAP121 on the Outer Mitochondrial Membrane Controls Hypoxia-Induced Mitochondrial Dynamics in Neurons

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1. Introduction

Mitochondria are highly dynamic organelles that continuously move, divide and fuse in a highly regulated fashion under the control of the so-called “mitochondria-shaping” protein family (Song et al., 2009; Meeusen et al., 2006; Olichon et al., 2003; Hales and Fuller, 1997, Santel and Fuller 2001, Zhao et al., 2011, Palmer et al., 2011, Yoon et al., 2003, Sesaki and Jensen, 1999; Bleazard et al., 1999; Mozdy et al., 2000). Neurons are particularly sensitive and vulnerable to abnormalities in mitochondrial dynamics, due to their large energy demand and their long extended processes where mitochondria need to be transported (Itoh et al., 2013, Bertholet et al., 2015, Bhat et al., 2015, Burtè et al., 2015; Zorzano and Claret, 2015).

Emerging evidence indicates a role for mitochondria-shaping proteins in several physiological functions, from apoptosis to ROS production, which are implicated in the pathogenesis of neurodegenerative diseases.

Moreover, changes in the mitochondrial shape can affect a variety of biological processes (da Silva et al., 2014, Yu et al., 2006; Li et al., 2004; Romanello et al., 2010). Mitochondria change their morphology by undergoing fusion or fission, and the fine balance between these two opposing reactions can be altered by a variety of factors including oxidative stress and apoptosis. Although mitochondria are key organelles for all cells, neurons are extremely sensitive to their functionality. Neurons are highly specialized cells with long extended processes including axons and dendrites. In addition, the long extended neuronal processes are highly active in intercellular signal transduction through the release of neurotransmitters from the synapses, a process that requires large amounts of energy.

Accordingly, the ability of mitochondria to fuse, divide and migrate is particularly important for synaptic function. In addition to energy supply, mitochondria also play a critical role in synaptic plasticity through the maintenance of calcium homeostasis in the synaptic microenvironment. Thus, mitochondria are important regulators of neuronal cell life and death via their role in energy production, Ca^{2+} signaling and participation in apoptosis (Yuan and Yanker, 2000).

Moreover, mitochondrial fission is an early step in the mitochondrial apoptosis pathway, and inhibiting fission can block or delay apoptosis in different cell types, including neurons. The balance between fusion and fission requires a tight control of proteins whose activity can be modulated by covalent modifications, such as phosphorylation, sumoylation and ubiquitylation (Carlucci et al., 2008; Chang and Blackstone, 2007; Cribbs and Strack, 2007; Han et al., 2008; Harder et al., 2004; Nakamura et al., 2006; Wasiak et al., 2007). Three proteins have been shown to be central to the fusion of mammalian mitochondria: Mitofusin 1 and 2 (Mfn1, Mnf2) and OPA1. Mfn1 and Mfn2, are essential GTPases localized to the mitochondrial outer membrane (Chen et al., 2003; Santel et al., 2001). OPA1 is localized to the intermembrane space, with tight association with the inner membrane (Griparic et al., 2004; Satoh et al., 2003).

A separate machinery mediates mitochondrial fission: Fis1, a mitochondrial outer membrane protein, and Drp1, another dynamin-related GTPase (Chen et al., 2005). In this respect, it has been shown that Drp1 is a direct substrate of PKA. The β-adrenergic stimulation by isoproterenol or PKA activation increases phosphorylation of Drp1 within the GTPase effector domain (GED) at Ser637 and inhibits its GTPase activity (Cribbs and Strack, 2007). On the other hand, membrane depolarization or L-type calcium channel agonists promotes de-phosphorylation of Drp1 by the calcium-activated Ser/Thr

phosphatase calcineurin (also known as PP2B) (Chang et al., 2007; Cribbs and Strack, 2007) and induce mitochondrial fragmentation and cell death (Cribbs et al., 2007). These findings indicate a mechanism whereby distinct intracellular second messengers can integrate and focus at target sites to regulate mitochondria dynamics and cell survival. Recent findings demonstrated that the mitochondrial PKA anchoring protein AKAP121 is rapidly degraded in cells exposed to hypoxia by a mechanism mediated by the activation of the E3 ubiquitin ligase SIAH2 (Carlucci et al., 2008). Moreover, is has also reported that AKAP12 interacts with the isoform 3 of the Sodium calcium exchanger, NCX3, on the outer mitochondrial membrane and that this interaction is crucial for mitochondrial calcium extrusion both in physiological and in hypoxic conditions (Scorziello et al., 2013).

Because mitochondrial dysfunction is considered to be one of the key event linking ischemic/recirculation insult with neuronal cell death (Berridge et al. 2003), and considering that the molecular mechanisms responsible for the regulation of mitochondrial dynamics in pathological conditions are only partially demonstrated, the present study was addressed to explore the role played by AKAP121 and NCX3, two proteins which affect mitochondrial function, in the molecular events underlying mitochondrial fusion and fission during ischemia/reperfusion.

1.1. Mitochondrial dynamics

Besides their role in energy production, mitochondria can act as sensors of metabolic homeostasis and regulate levels of intracellular signaling molecules. Among these, mitochondria participate to the handling of Ca^{2+} ions, which are involved in a variety of processes, such as ATP generation and neurotransmitters release (Tait and Green, 2012). Furthermore, these organelles are also involved in the intrinsic apoptotic pathway, which is triggered in response to different types of intracellular stress. Apoptosis brings to cell death via mitochondrial membranes permeabilization, release of cytochrome-c (Cyt-c) and subsequent caspases activation. All of these functions, which make mitochondria crucial for cell life and death, are strongly dependent on a set of processes encompassed into mitochondrial dynamics (da Silva et al., 2014). These processes are mitophagy, mitochondrial transport, fusion and fission, that, respectively, regulate mitochondrial turnover, mitochondrial migration throughout the cell and mitochondrial morphology (Chen and Chan, 2009). As a result, mitochondria can readily adapt to changes, in physiological or pathological cellular requirements. When mitochondrial dynamics are disrupted, cellular dysfunction occurs (Chen and Chan, 2009).

In the last decade, the knowledge about the mechanisms regulating mitochondrial dynamics has expanded. Although considerable advances have been made in the field of mitochondrial dynamics in recent years, the molecular mechanisms involved in their regulation remain to be elucidated (Zorzano and Claret, 2015).

As expected from their physiological relevance, alterations of fusion or fission are associated to pathological conditions, notably neurodegenerative diseases. Defects in mitochondrial dynamics have been associated to Alzheimer's, Parkinson's and Huntington's

diseases (AD, PD and HD) (Hroudova et al., 2014; Itoh et al., 2013a; Reddy, 2014; Van Laar and Berman, 2013), and mutations in genes encoding proteins essential for mitochondrial fusion are linked to Charcot–Marie–Tooth Disease and Dominant Optic Atrophy (Delettre et al., 2000; Zuchner et al., 2004). Other pathological conditions, such as hypoxia, modifies mitochondrial morphology (Peng et al., 2015; Dong et al., 2015). Therefore, the study of the mechanisms regulating mitochondrial dynamics could help the understanding of the molecular events that bring to neuronal death in neurodegenerative diseases.

1.1.1. Mitochondrial Fusion and Fission

The balance between fusion and fission determines mitochondrial morphology and requires specific proteins and phospholipids (Zhang et al., 2014). When fusion prevails on fission, mitochondria appear as an interconnected network of filaments, while they turn into small fragments when fission takes over. In contrast to numerous cells types and to the soma of neurons, where mitochondria are highly branched and interconnected, the mitochondria in neurites appear mainly as short filaments or dots. It is important to note that fusion/fission dynamics are also linked to mitochondrial mobility and positioning. Indeed, defects in fusion/fission and the consequent alterations of mitochondrial morphology affect intracellular distribution of mitochondria. Moreover, function and structure of mitochondria are also intimately linked. In fact, mitochondrial morphology can change depending on cellular requirement, and alterations of the proper shape of mitochondria compromise their functionality (Chen and Chan, 2009).

One of the most fundamental roles of fusion is to enable functional complementation among mitochondria through exchanges of proteins, like those of respiratory complexes, and mitochondrial DNA (mtDNA) nucleoids (Yang et al., 2015; Legros et al., 2002; Legros et al., 2004; Wilkens et al., 2013). Accordingly, loss of mitochondrial fusion is particularly deleterious for mtDNA integrity, as it results in increased mutation rate and genome loss (Amati-Bonneau et al., 2008; Chen et al., 2010; Hudson et al., 2008; Pelloquin et al., 1998, 1999). On the other hand, mitochondria undergo to fusion in response to cellular stress in order to stimulate ATP production and to protect the cell (Gomes et al., 2011; Tondera et al., 2009).

Mitochondrial fission is essential for the maintenance of mitochondrial morphology and distribution of mtDNA-nucleoids (Ban-Ishihara et al., 2013; Ishihara et al., 2015; Murley et al., 2013; Parone et al., 2008). Thus, emerging data in both yeast and mammals indicate that the majority of mitochondrial fission sites are located near mtDNA-nucleoids (Ban-Ishihara et al., 2013; Murley et al., 2013). Fission also enables the generation of smaller mitochondria that can be engulfed by autophagosomes and the isolation of damaged portions, like those with a reduced membrane potential (Twig et al., 2008; Legros et al., 2002; Sauvanet et al., 2012).

Mitochondrial dynamics, by modulating mitochondrial shape and the distribution of proteins in the mitochondrial membranes, further affect their permeability and the activation of apoptosis (Renault et al., 2015; Weaver et al., 2014). Several studies, indeed, have revealed functional and physical interactions between the proteins involved in fusion or fission events and members of Bcl-2 family responsible for the OMM permeabilization (Martinou and Youle, 2011). However, the exact mechanisms by which mitochondrial fusion and fission proteins influence OMM permeabilization are not clearly understood. In

addition, mitochondrial dynamics contribute to the regulation of cell proliferation (Mitra, 2013), modulate nutrients utilization and energy metabolism (Liesa and Shirihai, 2013; Zorzano et al., 2015), and are involved in the transition from a pluripotent to a differentiated state (Folmes et al., 2011; Kasahara and Scorrano, 2014).

The main proteins responsible for mitochondrial fusion and fission belong to the same family of Dynamin-Related-Proteins that possess several identifiable regions (Fig. 1): a highly conserved GTPase domain, two helical regions named middle domain (MD) and a GTPase effector domain (GED). These last two regions function together to mediate self-assembly, GTP hydrolysis and membrane remodeling (Ferguson and De Camilli, 2012; Praefcke and McMahon, 2004). Most of factors involved in mitochondrial dynamics appear conserved in almost all eukaryotes. However, these processes require numerous additional factors that are differently regulated between vertebrates, invertebrates and fungi (Zhao et al., 2013).

The main factors that drive the fusion of OMM are the mitofusins (Fig. 1B). They are GTP-binding proteins of the dynamin-superfamily conserved in all eukaryotes excepting plants (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Rojo et al., 2002; Logan, 2010; Roy et al., 2015). Lower eukaryotes and invertebrates encode a single homolog, Fzo1p in yeast, while vertebrates express two homologs, mitofusins Mfn1 and Mfn2, with high sequence identity (Rojo et al., 2002). Both mitofusins are ubiquitously expressed, but their expression levels and mRNA differ among tissues (Kawalec et al., 2014; Rojo et al., 2002). The presence of a transmembrane domain, that spans the OMM twice leading both N-terminal and C-terminal domains oriented towards cytosol (Rojo et al., 2002), distinguishes MFN/Fzo proteins from other members of the dynamin superfamily that are soluble and associate to mitochondrial membranes in a reversible

manner (Praefcke and McMahon, 2004). Studies conducted on cells lacking of one of both mitofusins demonstrated that Mfn1 and Mfn2 are needed on both mitochondria destined to fuse and that their functional GTP-binding domain is essential for the mitochondrial

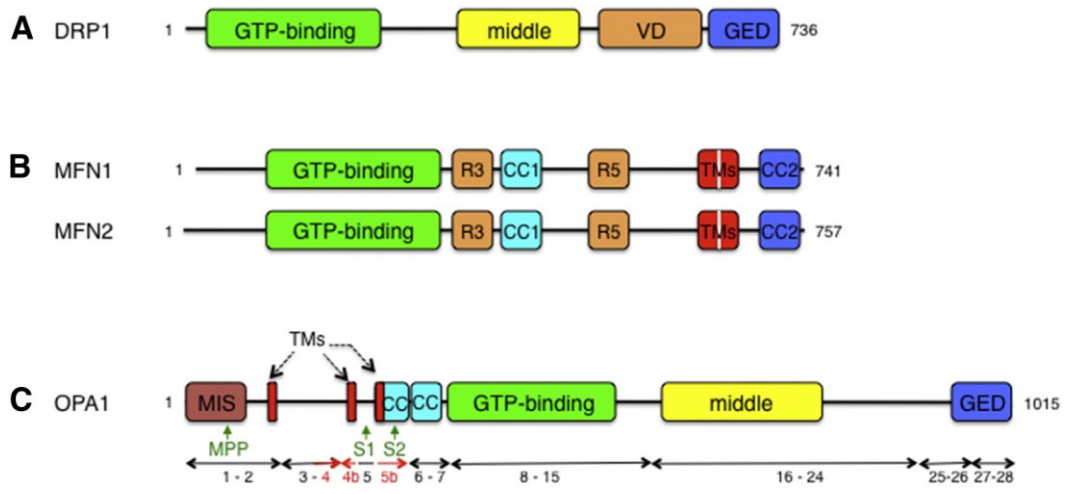

Fig. 1: Structural features of the main proteins involved in mitochondrial fusion/fission dynamics (Bertholet et al., 2015)

fusion. Furthermore, deletion of Mfn1 or Mfn2 strongly reduces mitochondrial fusion, but the absence of both mitofusins fully abolishes fusion (Chen et al., 2003; Koshiba et al., 2004). Study performed with mitofusin-mutants in cultured cells suggest a role of mitofusins in membrane tethering, the step preceding membrane docking and fusion (Koshiba et al., 2004).

The activity and amount of MFN/Fzo are modulated by several mechanisms. In mammals, mitochondrial depolarization induced through uncouplers inhibits fusion and stimulates mitophagy with concomitant Parkin-dependent ubiquitination and consequent degradation of Mfn1 and Mfn2 (Gegg et al., 2010; Tanaka et al., 2010). However, inhibition of a de-ubiquitinase enzyme in mammals stabilizes Mfn2 and stimulates Mfn2-dependent fusion

(Yue et al., 2014). Furthermore, mitofusin-dependent fusion has also been shown to be modulated by phosphorylation (Chen and Dorn, 2013; Leboucher et al., 2012, Pyakurel et al., 2015) and Bcl-2 family proteins (Hoppins et al., 2011). In addition, mitofusins expression and mitochondrial dynamics appeared altered in cells devoid of Bax and Bak (Karbowski et al., 2006). By contrast, the same morphological alterations were not similar in another study (Brooks et al., 2007) and both Bax and Bak appeared dispensable for stress-induced hyperfusion (Tondera et al., 2009).

In mammals, Mfn1 and 2 play also a relevant role in mtDNA maintenance. In fact, the lack of either or both mitofusins, which respectively reduces or abolishes mitochondrial fusion, provokes defects in mtDNA maintenance and in oxidative phosphorylation (Chen et al., 2010). In addition, mitofusins have been directly involved in the regulation of other cellular processes, including homeostasis of metabolites/co-factors like coenzyme Q and glucose as well as in insulin signaling (Mourier et al., 2015; Sebastian et al., 2012; Tubbs et al., 2014) and cell proliferation (Chen et al., 2014). In vivo and ex vivo studies further demonstrated that Mfn2 can modulate some functions of the ER unfolded protein response (UPR) (Debattisti et al., 2014; Munoz et al., 2013; Sebastian et al., 2012). It was proposed that these functions were mediated by the formation of ER-mitochondrial tethers (de Brito and Scorrano, 2008), but recent reports have questioned the role of Mfn2 in ER-mitochondrial tethering (Cosson et al., 2012; Filadi et al., 2015). Last but not least, studies in cultured neurons and in living zebra-fish revealed the contribution of mitofusins, and thus fusion, to mitochondrial movements. Indeed, Mfn2 was found to interact with Miro-proteins and its mutation was shown to hamper mitochondrial transport in axons (Chapman et al., 2013; Misko et al., 2012).

The fusion of OMMs is almost simultaneous and is linked to that of IMMs (Liu et al., 2009). The dissociation of fusion events allowed to reveal that MFN/Fzo is required for OMM fusion while OPA1/Mgm1p (Fig. 1C) is the main protein required for IMM fusion (Meeusen et al., 2006; Song et al., 2009). OPA1 is a human homolog of the fusogenic yeast proteins Mgm1p and Msp1p. OPA1 is ubiquitously expressed and it is located into inter-membrane space (IMS), anchored to IMM. The sorting of this GTPase is determined by an N-terminal mitochondrial import sequence (MIS) which is removed during the import into mitochondria. The MIS sequence is followed by three predicted transmembrane helices located before the GTPase domain (Guillou et al., 2005; Herlan et al., 2003; Olichon et al., 2002; Wong et al., 2000). OPA1 is further processed within mitochondria in steady state (Herlan et al., 2003; Leroy et al., 2010; Song et al., 2007; Guillou et al., 2005) and stress conditions (Sauvanet et al., 2012; Duvezin-Caubet et al., 2006; Griparic et al., 2007; Guillery et al., 2008; Ishihara et al., 2006; Song et al., 2007). Moreover, it was recently found that OPA1 function can be tightly regulated by ubiquitination, O-GlcNAcylation and acetylation/deacetylation (Kar et al., 2010; Makino et al., 2011; Samant et al., 2014).

The characterization of the role of OPA1, and its isoforms, in IMM fusion is still not clearly understood. Conversely to Mgm1p (Meeusen et al., 2006), the presence of OPA1 is only needed on one of the two mitochondria destined to fuse (Song et al., 2009); this may be due to its capacity to protrude the IMMs of fusing mitochondria towards each other (Ban et al., 2010). Of note, depending of its levels, OPA1 can induce “transient fusion”, that results in rapid exchange of soluble components without affecting the morphology of mitochondria, or a “complete fusion”, that allows the exchange of all mitochondrial components and that alters mitochondrial morphology (Liu et al., 2009).

Furthermore, particular OPA1 splice variants have an active anti-apoptotic activity (Cipolat et al., 2004; Olichon et al., 2007a). This function is attributed to complexes containing OPA1-oligomers that maintain the structure of the cristae junctions and sequester Cyt-c within the cristae (Frezza et al., 2006; Yamaguchi et al., 2008). Upon apoptosis, OPA1 complexes rapidly dissociate, leading to gradual release of Cyt-c from the cristae to the IMS and, after OMM permeabilization, from the IMS to the cytosol. While numerous results indicate that OPA1 assembly and disassembly regulate Cyt-c mobilization, controversies still persist concerning the relationships between cristae remodeling, disassembly of OPA1 oligomers, and Cyt-c mobilization (Scorrano, 2009; Scorrano and Korsmeyer, 2003; Yamaguchi and Perkins, 2009). Interestingly, Cyt-c mobilization was shown to be dependent on the interaction of BNIP3, a pro-apoptotic member of the Bcl-2 family, with OPA1 (Landes et al., 2010a).

The role of OPA1 in oxidative phosphorylation has long been known (Belenguer and Pellegrini, 2013) and the importance of this role was recently emphasized by data showing that OPA1 overexpression ameliorates the phenotype of two disease mouse models of defective mitochondrial respiratory chain (Civiletto et al., 2015). Indeed, OPA1-depleted cells display a severe reduction of endogenous respiration and a decrease of oxygen consumption driven by complexes I, II and IV (Chen et al., 2005). This could originate from mitochondria respiratory chain alterations probably linked with the disorganization of the IMM structure that occurs in OPA1 depleted cells (Griparic et al., 2004; Olichon et al., 2003). In this framework, it was also shown that the levels of mitochondria respiratory chain subunits are decreased upon OPA1 downregulation (Bertholet et al., 2013). Moreover, there is also evidence of physical interaction between OPA1 and the respiratory chain complexes I, II and III (Zanna et al., 2008). Furthermore, a recent work unraveled the

role of OPA1 in assembly of respiratory chain supercomplexes that increase the respiratory efficiency (Cogliati et al., 2013). Accordingly, a recent work with mouse models of mitochondrial diseases showed that OPA1 overexpression ameliorates their phenotype by correcting cristae ultrastructure and mitochondrial respiration and by increasing the levels and the assembly of the respiratory complexes (Civiletto et al., 2015). Thus, both metabolic and anti-apoptotic roles of OPA1 depend on its cristae-shaping activity and appear to be independent of its fusogenic function, which would be crucial for the quality control of mitochondria by allowing their complementation.

The major actor of mitochondrial fission is a cytosolic Dynamin-Related Protein (Fig. 1A), called Drp1 in mammals or Dnm1p in yeast (Sesaki et al., 2014). This protein and its role are conserved in all eukaryotes characterized to date (Sesaki and Jensen, 1999; Smirnova et al., 2001; Aldridge et al., 2007; Labrousse et al., 1999; Logan, 2010; Roy et al., 2015). Like most fusion/fission factors, Drp1 is ubiquitously expressed in mammals. This protein also mediate fission of peroxisomes (Schrader, 2006; Schrader et al., 2011). Visualization of the mitochondrial fission process revealed that soluble yeast Dnm1p and mammalian Drp1 reversibly associate to OMM at sites of mitochondrial division (Sesaki and Jensen, 1999; Smirnova et al., 2001), and that fission occurs preferentially at regions in contact with the endoplasmic reticulum (ER) (Friedman et al., 2011). In mammals, FIS1, MFF, MIEF1 and MIEF2 have been identified as proteins which mediate the association of Drp1 to mitochondria (James et al., 2003; Yoon et al., 2003; Gandre-Babbe and van der Bliek, 2008; Palmer et al., 2011; Zhao et al., 2011).

Numerous biochemical and structural studies have been conducted to find out the mechanism by which Drp1 induces mitochondrial fission. Similar to other members of the

dynamin-superfamily, Drp1/Dnm1p oligomerizes on OMM and constricts membranes upon GTP hydrolysis (Frohlich et al., 2013; Mears et al., 2011; Richter et al., 2015).

Mitochondrial recruitment, assembly, activity and stability of Drp1 are regulated by several post-translational modifications, including phosphorylation, S-nitrosylation, ubiquitination, SUMOylation and O-GlcNAcylation (Cribbs and Strack, 2007; Chang and Blackstone, 2007; Cereghetti et al., 2008; Han et al., 2008; Kashatus et al., 2011; Taguchi et al., 2007; Braschi et al., 2009; Figueroa-Romero et al., 2009; Zunino et al., 2009; Chang and Blackstone, 2010; Wilson et al., 2013). These events modulate mitochondrial fission in response to cellular needs. For examples, Drp1 recruitment to mitochondria is enhanced by its phosphorylation mediated by CDK1 kinase and leads to mitochondrial fragmentation as soon as cells enter mitosis (Kashatus et al., 2011; Taguchi et al., 2007).

The inactivation of Drp1 by knockout, or expression of dominant negative mutants provokes an increase in the length and interconnectivity of mitochondria in various mammalian cell lines (Bleazard et al., 1999; Otsuga et al., 1998; Pitts et al., 1999; Sesaki and Jensen, 1999; Smirnova et al., 2001). Moreover, knockdown of Drp1 in cultured cells leads to several defects, including lowered mitochondrial oxidative phosphorylation and inhibition of cell proliferation (Benard et al., 2007; Parone et al., 2008). Surprisingly, general knockout of Drp1 in mice revealed that fission is dispensable for cell growth and division as well as for mitochondrial oxidative phosphorylation, but essential for embryonic development (Ishihara et al., 2009; Wakabayashi et al., 2009).

Drp1 mediated-mitochondrial fission is also required for the segregation of dysfunctional mitochondria and for their removal through mitophagy (Twig et al., 2008). On the other hand, starvation was shown to induce mitochondrial elongation owing to the

phosphorylation of Drp1, which down-regulates its activity and spares mitochondria from autophagic degradation and stabilizes cellular ATP level (Gomes et al., 2011).

Several experimental systems revealed that apoptotic OMM permeabilization is accompanied by the fragmentation of mitochondria, but the role of Drp1-mediated fission remains debated (Arnoult, 2007). The recruitment of Drp1 upon apoptosis was linked to Bax/Bak proapoptotic proteins since Drp1 co-localized with Bax (Karbowski et al., 2002). Moreover, Drp1 association to OMM was promoted by Bax/Bak-dependent SUMOylation (Wasiak et al., 2007). Contrasting with earlier studies proposing that fission is required for apoptosis (Frank et al., 2001; Lee et al., 2004), later studies showed that downregulation of Drp1 induced a selective delay of Cyt-c release but did not inhibit efflux of Smac/Diablo or apoptotic cell death (Estaquier and Arnoult, 2007; Parone et al., 2006). Later on, studies on mouse-embryonic fibroblasts (MEF) devoid of Drp1 confirmed that Drp1-dependent mitochondrial fission is dispensable for the apoptotic permeabilization of mitochondria, but can facilitate this process in some cell lineages (Ishihara et al., 2009; Wakabayashi et al., 2009).

1.2. Mitochondrial fusion/fission in neurodegenerative diseases

Several neurodegenerative diseases are associated to different mitochondrial defects that affect oxidative phosphorylation, production of reactive oxygen species, mobility, clearance by mitophagy and fusion/fission dynamics (Fig. 2) (Burte et al., 2015; Dupuis 2014; Ryan et al., 2015; Van Laar and Berman, 2013; Yan et al., 2013). Indeed, pathogenic mutations in the leucine-rich repeat kinase 2 (LRRK2), huntingtin and sacsin molecular chaperone result in distinct neurodegenerative phenotypes such as: autosomal dominant PD (Paisan-Ruiz et al., 2004), Huntington disease (Macdonald et al., 1993) and autosomal recessive spastic ataxia of Charlevoix–Saguenay, ARSACS (Engert et al., 2000). All these mutated proteins have been shown to potentiate the pro-fission activity of Drp1, both in vitro and in vivo, suggesting a strong link between altered mitochondrial dynamics and neurodegeneration (Costa et al. 2010; Song et al., 2011; Girard et al., 2012; Wang et al., 2012; Shirendeb et al., 2011; Shirendeb et al., 2012). Furthermore, synaptic degeneration was also observed in patients with AD and in neurons obtained from mouse models of AD as result of impaired mitochondrial biogenesis, defective axonal transport of mitochondria and increased Drp1-mediated mitochondrial fission (Manczak et al., 2011; Manczak and Reddy, 2012; Calkins et al., 2011).

The correlation between abnormalities in mitochondrial dynamics and neurodegenerative diseases is also suggested by the fact that mutations in genes involved in fusion/fission have been linked to two groups of neuropathies, Dominant Optic Atrophy (DOA) and Charcot–Marie–Tooth disease (CMT). DOA is mainly characterized by a degeneration of the optic nerves causing loss of visual acuity and abnormalities of color vision (Lenaers et

al., 2012). This disease affects primarily the retinal ganglionic cells, RGC, whose axons form the optic nerve. DOA shows variable expression ranging from asymptomatic state to

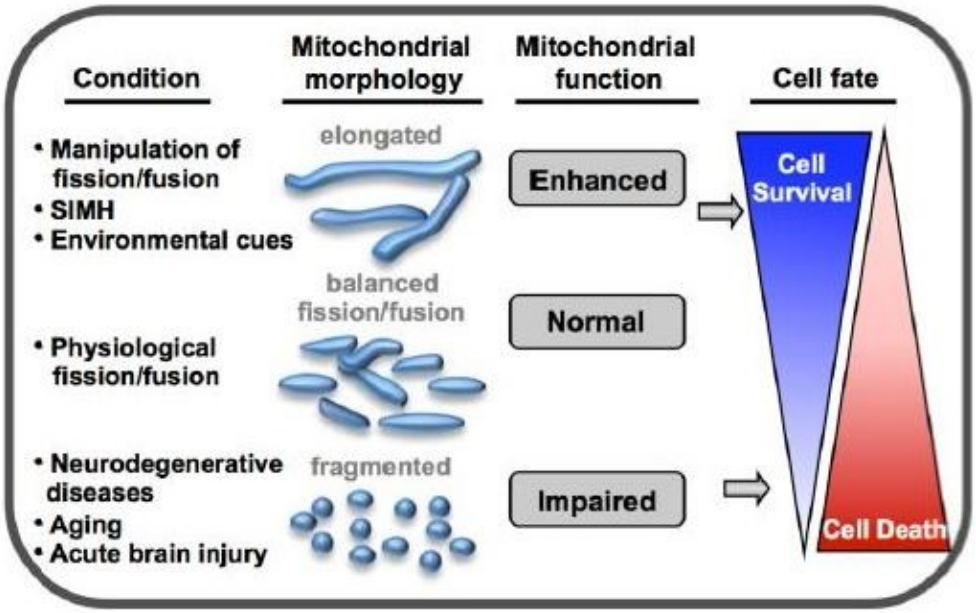

Fig. 2: Mitochondrial conditions in cell survival and cell death (Khacho and Slack, 2015).

blindness. 60–80% of DOA patients have mutation in the OPA1 gene (Amati-Bonneau et al., 2009). More than 200 different OPA1 mutations have been reported, among which missense, frameshift, nonsense and deletion occur, respectively, in 26%, 16%, 14% and 7% of the total cases (Ferre et al., 2015). Although optic nerve degeneration remains the main feature of DOA, up to 20% of patients with OPA1 mutations also develop extra-ocular neurological complications, including deafness, ataxia, peripheral neuropathy, myopathy and progressive external ophthalmoplegia (Yu-Wai-Man et al., 2010a). Furthermore, a recent work reports the association of parkinsonism with OPA1 mutations, suggesting a direct link between mitochondrial dynamics and age-related neurodegenerative diseases (Carelli et al., 2015).

Several DOA mouse models have been obtained in the last years (Alavi et al., 2007; Davies et al., 2007; Sarzi et al., 2012), bearing heterozygous OPA1 mutations either on the GTPase or GED domains that provoke a decrease in the protein quantity, while homozygous mutations are embryonic lethal. These mice show many pathological features, including mild age-dependent ocular phenotypes with evidence of RGC loss or dysfunctions, as well as degenerative features in the optic nerve including demyelination and axonal degeneration (Williams et al., 2011). Nonetheless, the exact mechanism by which inactivation of OPA1 lead to neurodegeneration still has to be clarified. Impairments of mitochondrial morphology and functions as well as increased autophagy and apoptosis appear to be involved (Ban et al., 2010; Agier et al., 2012; Amati-Bonneau et al., 2005; Chevrollier et al., 2008; Olichon et al., 2007b; Zanna et al., 2008; Davies et al., 2007; White et al., 2009; Yu-Wai-Man et al., 2010b). In addition, OPA1 was shown to protect neurons from excitotoxicity (Jahani-Asl et al., 2011; Kushnareva et al., 2013; Nguyen et al., 2011).

Like DOA, also CMT disease can be related to mutations in a gene involved in mitochondrial fusion. CMT disease includes a heterogeneous group of hereditary motor and sensory neuropathies, (HMSN), that can be divided in two main groups, demyelinating CMT1 and axonal CMT2. The former group is associated with mutations in genes required for myelination, whereas the latter is mainly due to mutations in genes essential for the maintenance of axonal structure and function (Di Vincenzo et al., 2014; Murphy et al., 2012; Gentil and Cooper, 2012). The main form of axonal CMT2, occurring in about 20% of patients, is the autosomal dominant CMT2A, in which the most common mutations are those of Mfn2 (Verhoeven et al., 2006; Zuchner et al., 2004). The first symptoms of the disease, characterized by muscle weakness, hyporeflexia and sensory loss in the lower

limbs as well as gait defects, appear before the age of 10 for the most of patients (Lawson et al., 2005; Verhoeven et al., 2006). Missense mutations represent the large majority and are distributed throughout the entire ORF sequence, though most of these mutations have been found in the functional domains (Bombelli et al., 2014; Chung et al., 2006; Di Vincenzo et al., 2014; Feely et al., 2011; McCorquodale et al., 2011; Verhoeven et al., 2006). A subset of Mfn2 mutations is also associated to visual failure and optic atrophy thus resembling the DOA phenotype associated to some OPA1 mutations (Rouzier et al., 2012; Yu-Wai-Man et al., 2010a). Thus, not only both severe CMT2A and DOA are caused by mutations in genes regulating mitochondrial fusion, but they also share some similar clinical features. However, the fact that the Mfn2 protein is expressed ubiquitously while the symptoms are restricted to neurons indicates that Mfn2 exerts some neuron-specific functions (Kawalec et al., 2014).

Brain ischemia is another pathological condition in which an alteration of mitochondrial morphology has been observed. Indeed, in a study conducted in rats subjected to transient global ischemia it has been described an elongation of mitochondria in hippocampal neurons 7 days after ischemia/reperfusion (Bertoni-Freddari et al. 2006). In addition, brain ischemia increased OPA1 and FIS1 while a prolonged reperfusion causes raise of phosphorylated Drp1 expression in the ischemic penumbra, suggesting that an activation of both mitochondrial fission and fusion mechanisms take place in this pathological condition (Liu et al., 2012a; Liu et al., 2012b). Accordingly, a recent study conducted by Wappler and colleagues demonstrated that changes in mitochondrial morphology resembling fission and fusion occur in rat primary neuronal cultures exposed to hypoxia. In fact, the exposure of neurons to 3 hours of oxygen and glucose deprivation, OGD, decreased Drp1 expression and increased Mfn1 levels. Nonetheless, Drp1 does not seem to be a key regulator of

mitochondrial adaptation to hypoxia, given that its blockage did not reduce cell death or alter mitochondrial morphology following OGD (Wappler et al., 2013). By contrast, Grohm et al. showed that Drp1 is directly involved in the modulation of mitochondrial dynamics in case of hypoxia. In fact, the reduction of mitochondrial fission through the knocking down or the pharmacological inhibition of Drp1 are, respectively, able to reduce the toxicity in a glutamate-induced oxidative stress model in HT22 cells and the infarcted area in a mouse model of transient focal ischemia (Grohm et al., 2012).

Given that the molecular mechanisms of mitochondrial dynamics in diseases are still unclear, further studies are needed to fully understand the role of these phenomena in pathological conditions such as hypoxia/reoxygenation.

1.3. The hypoxia-induced mitochondrial dysfunction

Mitochondrial dysfunction is a common feature of several neurodegenerative diseases. The importance of mitochondria is due to their central role in the regulation of several processes crucial for cell death or life. Because mitochondria generate more than 90% of cellular ATP, they efficiently supply energetic needs of cells. ATP production is mainly accomplished through the oxidative phosphorylation, a process that occurs at IMM level where the respiratory chain is located. In these process, electrons are transferred from reduced NADH molecules, deriving from Krebs cycle, to oxygen via various protein complexes constituting the respiratory chain. The electrons flux is paralleled by the

transfer of H^+ from mitochondrial matrix to IMS, which produces an electrochemical gradient among the two IMM sides. This gradient generates the mitochondrial membrane potential, $\Delta\Psi_m$, whose maintenance is fundamental for the proper function of the respiratory chain.

Tissues with a high energy demand, such as liver, muscle and brain, are strongly dependent on mitochondria to accomplish their functions. Notably, human brain, which constitutes only the 2% of total body mass, consumes about the 20% of resting metabolic energy. This characteristics make neuronal cells very susceptible to ATP depletion (Silver and Erecinska, 1998). Furthermore, inside neurons, mitochondria are located in the regions of high metabolic demand, such as synapses and Ranvier nodes (Berthold et al., 1993; Bristow et al., 2002; Kageyama and Meyer, 1988; Rowland et al., 2000).

As mentioned before, mitochondria are very sensible to pathologic conditions. Brain ischemia, for instance, strongly affect mitochondrial function. Besides cancer and cardiac diseases, stroke is the primary cause of adult disability and death (Flynn et al., 2008; Doyle et al., 2008). Ischemic stroke, which is caused by an occlusion of an artery in the brain, represents the 80–85% of all cases of stroke. If blood flow is not rapidly restored brain tissue can be strongly and permanently damaged (Flynn et al., 2008). Given the high metabolic rate, brain tissues are particularly vulnerable to ischemic event which reduces oxygen and glucose delivering to the brain and, therefore, affects ATP production. As a consequence of ATP depletion, several neuronal functions, such as the maintenance of ionic gradients, are impaired (Martin et al., 1994). However, mitochondria play a central role in the pathogenesis of ischemic damage not only for their involvement in ATP generation but also for their role in the activation of cell death pathways (Sugawara et al., 1999; Wang et al., 2003).

The cascade of events leading to neuronal damage during ischemia is rather complex. One of the early consequence of ATP depletion in the ischemic brain is the depolarization of neuronal plasma membrane. The lack of ATP, indeed, impairs Na^+/K^+ exchanger activity inducing a depolarization of plasmamembrane. This depolarization opens voltage-gated channels and allows Ca^{2+} entry into axon terminal, which in turn induces the release of glutamate from pre-synapse (Choi et al., 1994; Sattler and Tymianski, 2000; Nishizawa, 2001). Glutamate, by acting on its receptors on post-synaptic side, causes a further increase in cytosolic Ca^{2+}. Likewise, the activity of calcium ATPase, which enables the maintenance of Ca^{2+} homeostasis, is strongly compromised by ATP impairment and Ca^{2+} ions are not efficiently removed from the cytosol. As a consequence, the increased cytosolic Ca^{2+} levels in neurons during ischemia expose mitochondria to micromolar Ca^{2+} concentrations which favor the accumulation of Ca^{2+} into the mitochondrial matrix. This Ca^{2+} uptake is due to the activity Ca^{2+} channels, and exchanger located on mitochondrial membranes. However, mitochondria are able to buffer Ca^{2+} and to face cytosolic overload within a threshold level. A moderate calcium entry, indeed, boosts the activities of enzymes of the tri-carboxilic cycle and thus cellular metabolism. Beyond this level, mitochondria are no longer able to physiologically store Ca^{2+} ions and undergo mitochondrial dysfunction (Nicholls and Chalmers, 2004). Indeed, an excessive increase in matrix Ca^{2+} concentration induces oxidative stress and alters the normal mitochondrial function and membranes permeability, allowing the release of pro-apoptotic factors (Starkov et al., 2004, Denton et al., 2009). Moreover, lactic acidosis, which arise from the anaerobic metabolism, causes an intracellular acidification that increases intracellular Na^+ as a result of Na^+/H^+ exchange iperactivity, which in turn leads to a further accumulation of Ca^{2+} into the citosol owing to the reversal of activity of Na^+/Ca^{2+} exchanger. All these

pathways bring to a rapid rise in intracellular Ca^{2+} that activates plasma membrane calcium pumps (PMCA), which aggravate cellular damage by dissipating ATP molecules. However, although the magnitude of intracellular calcium overload is comparable to that induced by the opening of membrane Ca^{2+}channels, the uptake of calcium induced by ischemia lasts much more and brings to mitochondrial dysfunction (Bano and Nicotera 2007; Cross et al., 2010, Scorziello et al., 2013).

The uptake of Ca^{2+} ions into mitochondria also depends on their subcellular localization. Indeed, mitochondria located near the plasma membrane are more subjected to calcium overload owing to failure of calcium extrusion mechanisms and the activation of voltage-gated calcium channels (Frieden et al., 2002; Frieden et al., 2005). Indeed, it has been demonstrated that mitochondria rapidly take up calcium coming from voltage-gated channels (Montero et al., 2000). Therefore, mitochondrial dysfunction induced by calcium overload during ischemia might be a direct consequence of glutamate excitotoxicity arising from over-activation of plasmamembrane NMDA receptors (Starkow et al., 2004; Pivovarova et al., 2010; Stanika et al., 2010; Stanika et al., 2009; Tymianski et al., 2007). This hypothesis place the over-activation of ionotropic glutamate receptors as a main cause of mitochondrial dysfunction, and suggests a “route specific” for neurotoxicity which depends more on the routes of calcium entry than on the magnitude of calcium overload (Pivovarova et al., 2010; Tymianski et al., 1993; Liu et al., 2007). On the other hand, the release of calcium from the endoplasmic reticulum, ER, has been also associated to ischemia-induced mitochondrial dysfunction (Pascen and Doutheil, 1999; Pisani et al., 2000; Chen et al., 2008). The bridging between mitochondria and ER mediated by specific linker proteins makes possible the interplay between these two cellular compartments (Cosordas at al., 2006; Cosordas et al., 2010). This interaction favors calcium uptake in

mitochondria adjacent to ER subsequent to the release of Ca^{2+} triggered by the opening of IP3 receptors (Giacomello et al., 2010; Rizzuto et al., 1993). Thus, mitochondrial calcium uptake during brain ischemia is a consequence of both Ca^{2+} entry across plasma membrane and Ca^{2+} release from ER.

The mechanism by which neuronal death occurs involves both necrotic and apoptotic pathways. However, originally it was believed that ischemia-induced cell death was exclusively due to necrosis as consequence of organelles and plasma membrane swelling. The scenario changed with the detection of DNA fragmentation and apoptotic bodies in brain tissue of ischemic rats, suggesting apoptosis as mechanism of ischemia-induced cell death (Li et al., 1995a; Li et al., 1995b) revitalizing the hypothesis of a mitochondrial dysfunction in the pathogenesis of the neuronal damage subsequent to the ischemic insult.

1.4. Mitochondrial calcium homeostasis

The maintenance of mitochondrial calcium homeostasis is fundamental to warrant a proper mitochondrial function. Therefore, considering that dysregulations of mitochondrial calcium concentration lead to mitochondrial dysfunction, a tight regulation of calcium homeostasis is constantly required. Mitochondria participate to the regulation of calcium homeostasis in concert with Ca^{2+} ATPase of sarco-endoplasmic reticulum and plasma membrane, and Na^{+}/Ca^{2+} exchanger, NCX (Saris and Carafoli 2005). Various mechanisms regulating the influx and efflux of Ca^{2+} are located in mitochondrial membranes (Fig. 3).

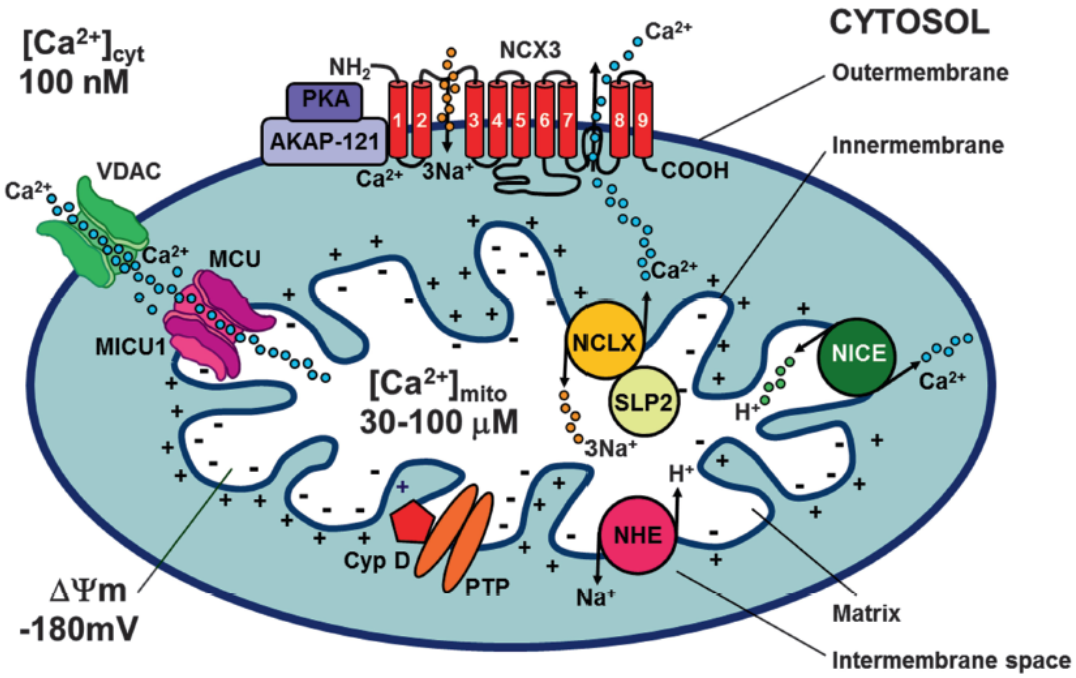

Fig. 3. Scheme of the mechanisms regulating mitochondrial calcium homeostasis: VDAC, Voltage Dependent Anion Channel; **NCX3**, Na^+/Ca^{2+} exchanger 3; **AKAP121**, A-Kinase Anchoring Protein 121; **MCU**, Mitochondrial Calcium Uniporter; **NCLX**, Na^+/Ca^{2+} Li^+-dependent Exchanger; **NICE**, Na^+-Independent Ca^{2+} Exchange; **NHE**, Na^+/H^+ Exchanger; **PTP**, Permeability Transition Pore; **PKA**, Protein Kinase A; **SLP2**, stomatin-like protein 2; **Cyp D**, cyclophilin D.

These mechanisms, become extremely important when the raise in the cytosolic concentrations of Ca^{2+} ions reaches levels of 400-500 nM and leads to mitochondrial Ca^{2+} uptake (Nicholls and Scott, 1980).

The influx of Ca^{2+} is mainly mediated by a recently identified mitochondrial Ca^{2+} uniporter, MCU, whose activity depends on $\Delta\Psi_m$ (De Stefani, 2011). In particular, $\Delta\Psi_m$ has to be maintained at very negative levels of about 180 mV (Bernardi et al., 1999). Likewise, the uptake of Ca^{2+} into mitochondria is also related to $[Ca^{2+}]_m$, which is kept low in physiological condition. MCU is a two transmembrane protein of 40 kDa located in the

IMM (De Stefani 2011). Besides MCU, that represents the main route by which Ca^{2+} ions are taken up into mitochondrial matrix, another pathway of Ca^{2+} uptake has been identified. This mechanism, known as rapid uptake mode, RaM, transfers Ca^{2+} ions in a very rapid way into the mitochondrial matrix (Sparagna et al., 1995; Buntinas et al., 2001). Surprisingly, it has been recently understood that also OMM takes part to the mechanisms regulating mitochondrial Ca^{2+} cycling. Notably, OMM acts as a barrier not only for Ca^{2+} uptake but also for Ca^{2+} efflux. The discovery of the voltage dependent anion channel, VDAC, supported the hypothesis that OMM plays an active role in Ca^{2+} homeostasis. VDAC, indeed, is located in OMM and regulates its permeability to Ca^{2+} ions. Furthermore, it was hypothesized that VDAC, by acting as a filter of Ca^{2+} at OMM level, modulates Ca^{2+} access to MCU (Crompton et al., 2002).

Concerning the mechanisms by which Ca^{2+} is extruded from mitochondria, two exchange pathways have been identified, the H^+/Ca^{2+} and Na^+/Ca^{2+} exchangers (Carafoli, 2003). The former, was defined Na^+-independent pathway for Ca^{2+} efflux, NICE, while the latter is Na^+-dependent pathway for Ca^{2+} efflux, NCE. A further mechanism of mitochondrial Ca^{2+} efflux is represented by the mitochondrial permeability transition pore, mPTP. The identity of mitochondrial Ca^{2+} transporters of NCE have been mostly identified in the last decade. One of these transporters is NCLX, a Li^+ sensitive protein of NCX family localized within the cristae of IMM and that participates to the mitochondrial Na^+/Ca^{2+} exchange (Palty et al., 2010). More recently, it was found that NCX3 is the only isoform of Na^+/Ca^{2+} exchanger localized into OMM where it plays a relevant role in regulating mitochondrial Ca^{2+} homeostasis in both basal and hypoxic conditions (Scorziello et al., 2013). These two Na^+/Ca^{2+} exchanger might be part of the same mechanism of Ca^{2+} extrusion from mitochondria. This pathway could occur in two consecutives steps. The first step is the

efflux of Ca^{2+} from matrix to IMS, while the second allows the extrusion of Ca^{2+} from IMS to cytosol. The efflux pathway NCE is relevant in excitable tissues, and is saturated at low $[Ca^{2+}]_m$, lower than 1μM. (Kim and Matsuoka, 2008). The role played by NCX3 in the regulation of $[Ca^{2+}]_m$ further supports the hypothesis that OMM is not a passive permeable membrane and acts as barrier for Ca^{2+} influx and efflux (Szabadkai and Duchen, 2008).

While NCE is a mechanism of Ca^{2+} extrusion typical of excitable tissues, the NICE occurs in not excitable cells. Regarding this pathway, no cations have been found to be exchanged with Ca^{2+}, but it is believed that a H^+/Ca^{2+} exchange occurs (Saris and Carafoli, 2005). This H^+/Ca^{2+} exchanger requires the maintenance of $\Delta\Psi_m$, operates with a very slow kinetic and is saturated by low Ca^{2+} concentrations (Bernardi et al., 1999).

Finally, mPTP also regulates Ca^{2+} efflux from mitochondria. The opening of mPTP is due to Ca^{2+} accumulation into mitochondria, and its composition has been recently clarified. For a long time it was believed that the mPTP was generated at the contact sites of the inner and outer membranes at the level of VDAC and the adenine nucleotide translocator, ANT (Beutner et al., 1996). However, this hypothesis was discarded because while mPTP is sensitive to cyclosporin A, VDAC and ANT are not (Kokoszka, et al., 2004; Krauskopf et al., 2006; Baines et al., 2007). More recent evidence, showed that mPTP forms from dimers of F_0F_1ATP synthase (Giorgio et al., 2013). In particular, it has been demonstrated that c-subunit ring of the F_0 of ATP synthase forms a voltage-sensitive channel, whose persistent opening leads to rapid and uncontrolled depolarization of the IMM in cells. Moreover, a long lasting Ca^{2+} accumulation into mitochondrial matrix enlarges the c-subunit ring, providing a mechanism for mPTP opening (Alavian et al., 2014). Regarding the physiological role of mPTP, its opening could represent a way to release pro-apoptotic

factors, to remove damaged molecules from matrix, or to allow a rapid Ca^{2+} release from mitochondria with a compromised $\Delta\Psi_m$ (Bernardi et al., 1999).

Thus, as described in this paragraph, several Ca^{2+} transporters involved in the regulation of mitochondrial Ca^{2+} homeostasis have been identified. Their presence in mitochondrial membranes is a proof of the mechanisms that tightly regulates $[Ca^{2+}]_m$. Among these, mNCX3 appears to be particularly important in regulating the levels of Ca^{2+} ions into mitochondrial matrix, especially in a pathological condition such as that evoked by ischemic events.

1.5. Role of NCX3 in the regulation of calcium homeostasis during hypoxia

NCX is a transmembrane proteins that exchange Na^+ with Ca^{2+} ions in a bidirectional way. For this reason, NCX is a protein involved in the regulation of the cellular homeostasis of these two ions. Three isoforms of NCX have been identified, NCX1(Nicoll et al., 1990), NCX2 (Li et al., 1994) and NCX3 (Nicoll et al., 1996b). NCX1 is expressed in several tissues, including brain, heart, skeletal and smooth muscle, kidney, eye, secretory, and blood cells, whereas transcripts encoded by the NCX2 and NCX3 genes have been found exclusively in neuronal and skeletal muscle tissues (Lee et al., 1994). All the isoforms share the same structure, that mainly consist of nine transmembrane segments and a big intracellular loop. Moreover, while the N-terminus protrudes on the extracellular side of the plasma membrane, C-terminus is into the cell (Nicoll et al., 1999). The three isoforms of NCX exchange three Na^+ ions with one Ca^{2+} ion, and therefore share the same stoichiometry (Fujioka et al., 2000; Hang and Hilgemann, 2004). Interestingly, NCX3 is

the only isoform of Na^+/Ca^{2+} exchanger which has been found at mitochondrial level (Scorziello et al., 2013). Before of NCX3, NCLX, which mediates Na^+- and Li^+-dependent Ca^{2+} efflux from the mitochondrial matrix to IMS, has been found on the cristae of IMM (Palty et al., 2010). However, from the discover of NCX its subcellular localization at mitochondrial level was debated for long time. Interestingly, evidence suggested that OMM serves as a permeability barrier not only to Ca^{2+} influx but also to Ca^{2+} efflux (Baines et al., 2007; Crompton et al., 2002; Moran et al., 1992), letting to hypothesize that a Na^+-dependent Ca^{2+} extruding mechanism could operate in removing Ca^{2+} from the intermembrane space to the cytoplasmic compartment. The finding of NCX3 on OMM and the study of its functional role confirmed this hypothesis. In particular, it has been found that NCX3 cooperates with AKAP121, a member of kinase A anchoring proteins family, AKAPs, in the extrusion of Ca^{2+} from mitochondria. Indeed, in baby hamster kidney cells, BHK, transfected with NCX3 and constitutively expressing AKAP121, $[Ca^{2+}]_m$ was lower than that measured in BHK-Wt cells and hypoxia-induced Ca^{2+} accumulation into mitochondria is prevented. Conversely, the silencing of AKAP121 induced by RNA interference reduced the extrusion of mitochondrial Ca^{2+} mediated by NCX3 and raised $[Ca^{2+}]_m$ in both BHK and neuronal cells. Last but not least, the extrusion of Ca^{2+} from mitochondria by NCX3 resulted in an increase in cell survival upon hypoxia (Scorziello et al., 2013). Accordingly, further evidence supported the important role played by NCX3 in the regulation of $[Ca^{2+}]_m$ during hypoxia. Indeed, it has been demonstrated that the exposure of cortical neurons to ischemic preconditioning, IPC, induced an increase in NCX3 expression an activity, and a consequent reduction of $[Ca^{2+}]_m$ after oxygen and glucose deprivation, OGD, followed by reoxygenation. This effect is prevented by treating neurons with CGP37157, a selective blocker of mitochondrial NCX. In addition, the

stimulation of NCX3 induced by IPC prevented the worsening of mitochondrial redox activity and favored neuronal survival after OGD plus reoxygenation (Sisalli et al., 2014). Thus, this recent evidence demonstrated that NCX3 is a key regulator of mitochondrial Ca^{2+} homeostasis, and that AKAP121 also plays an important role in maintaining $[Ca^{2+}]_m$ by supporting NCX3 activity at the OMM level.

1.6. AKAP proteins

Subcellular targeting through the interaction with anchoring proteins has emerged as an important mechanism by which signaling enzymes are localized close to their substrates. In this regard, AKAP proteins act as scaffold and concentrate protein kinase A, PKA, in specific subcellular compartments (Rubin et a., 1994; Dodge and Scott, 2000; Edward and Scott, 2000).

More than 50 proteins are grouped in AKAP family. These proteins are functionally related and structurally similar. They share a RII-binding site formed by an helix with 14-18 amphipathic amino acids on one side and charged residues on the other side, which binds to the amino termini of PKA-RII dimer (Carr et al., 1991; Newlon et al., 1997). Some AKAPs, instead, bind to RI subunit of PKA, but with a lower affinity compared to the binding between RII subunit. However, D-AKAP1 and D-AKAP2 are able to bind both the subunits of PKA.

AKAPs contains specific signal peptides in their amino acid sequence that determine their subcellular localization. Indeed, AKAPs proteins have been found in a various cellular

compartments, such as dendrites, ER, mitochondria, nuclear membrane, plasma membrane and vesicles (Wong and Scott, 2004).

Besides kinases, other enzymes, such as phosphatases and phosphodiesterases interact with AKAPs (Carlisle Michel et al., 2004).

1.6.1. Role of AKAP121 in the cellular hypoxia

Mitochondrial AKAP121, also known as D-AKAP1, allow the localization of PKA on OMM. This protein is widely expressed in several tissues such as germ-cell lineage, heart and thyroid, and its expression is regulated at the transcriptional level by the cAMP/PKA pathway (Feliciello et al., 1998). AKAP121, AKAP100 and AKAP84 are splice variants of the same gene, AKAP1 (Lin et al., 1995; Trendelenburg et al., 1996; Chen et al., 1997; Huang et al., 1997; Huang et al., 1999; Furusawa et al., 2002). These splice variants differ from each other in the C-terminus, whereas share a region of about 500 amino acid at N-terminus level. The structure of AKAP121 is composed of different domains (Fig. 4):

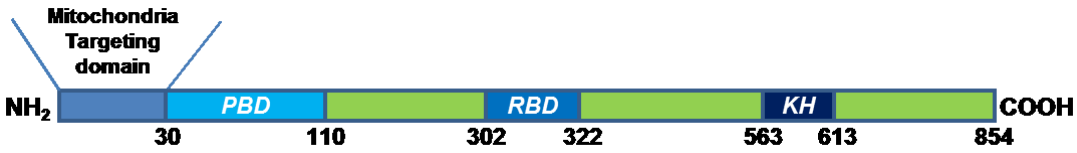

Fig. 4. AKAP121 structure: PBD, Phosphatase binding domain, which mediates the binding to PKA; **RBD**, R-binding domain, with RNA-binding capabilities; **KH**, KH domain, which binds to Tyrosine Phosphatase D1.

- R-binding domain (residues 302-322), which is identical in all the splice variants and mediates the binding with high affinity to RII subunit of PKA and with low affinity to RI subunit (Herberg et al., 2000);
- The first 30 NH_2-terminus residues, that determine the targeting of AKAP121/84-PKAII complexes to mitochondria in both male germ-cells and transfected heterologous cells (Lin et al., 1995; Chen et al., 1997);
- Phosphatase binding domain (residues 30-110), which binds to Tyrosine Phosphatase D1 (Moller et al., 1994; Cardone et al., 2004);
- KH domain (residues 565-613), with RNA-binding capabilities (Trendelenburg et al., 1996; Chen et al., 1997).

From a functional point of view, it has been showed that AKAP121 by anchoring PKA allows that cAMP signaling pathway reaches mitochondria and suppresses apoptosis (Harada et al., 1999). Indeed, the increase in cAMP following adenylate cyclase stimulation activates a membrane-anchored PKA, which in turn phosphorylates the serine 112 of the pro-apoptotic protein BAD. Unphopshorylated BAD binds to and inactivates anti-apoptotic Bcl-2 homologs, favoring the release of Cyt-c from mitochondria and the activation of apoptosis. As a consequence of PKA-mediated BAD phosphorylation, BAD association to Bcl-2 homologs is blocked and apoptosis inhibited (Harada et al., 1999).

Recently, it has been demonstrated that AKAP121 regulates the activity of components of mitochondrial respiratory chain, promoting mitochondrial membrane hyperpolarization and improving ATP synthesis in a PKA dependent manner (Livigni et al., 2006). Furthermore, AKAP121 is degraded during hypoxic conditions as consequence of the activation of the hypoxia-induced of the E3 ubiquitin-ligase Siah2 (Nakayama et al., 2004; Carlucci et al.,

2008). This enzyme leads to the ubiquitination of AKAP121 and to its proteasomal degradation in both *in vitro* and *in vivo* conditions.

The reduction of AKAP121 brings to an impairment of mitochondrial activity during hypoxia (Carlucci et al., 2008).

Besides its role in regulating mitochondrial respiratory activity, by anchoring PKA on OMM, AKAP121 influences also mitochondrial morphology. In particular, it has been demonstrated that AKAP121 is able to induce mitochondrial fusion both in a PKA dependent and in PKA independent manner (Kim et al., 2011). Indeed, on one hand AKAP121 induces Drp1 inhibition through its phosphorylation via PKA, and on the other hand, due to a specific domain localized in its central structure, AKAP121 hampers Drp1-Fis1 interaction on mitochondrial surface (Kim et al., 2011). It has been reported that the reduced availability of AKAP121 by Siah2 in cardiomyocytes exposed to hypoxia relieves Drp1 inhibition by PKA and increases its interaction with Fis1, resulting in mitochondrial fission (Kim et al., 2011). Accordingly to these data, it has been showed that cAMP accumulation in both neuronal and non-neuronal cells brings to the activation of PKA/AKAP121 pathway which in turn reshapes mitochondria into an interconnected network (Merrill et al., 2011). In particular, PKA-mediated Drp1 phosphorylation at Ser656, inhibits the disassembly step of its catalytic cycle, bringing to the accumulating of large and slowly recycling Drp1 oligomers on the OMM. This effect was prevented through the knocking down or the inhibition of PKA (Merrill et al., 2011).

2. Aim of the study

The purpose of the present study was to investigate whether NCX3 and AKAP121 are involved in the regulation of mitochondrial dynamics in an *in vitro* model of brain ischemia. In particular, two processes among mitochondrial dynamics were examined: mitochondrial fission and fusion.

The working hypothesis was suggested by the fact that, hypoxia reduces the expression of both NCX3 and AKAP121, two proteins important for mitochondrial Ca^{2+} homeostasis. Likewise, data obtained in our laboratory demonstrated that in hypoxic conditions mitochondrial fragmentation occurs. Therefore, in the aim of understanding whether the alterations of NCX3 and AKAP121 expression might be related to changes in mitochondrial dynamics during hypoxia, all the experiments were performed in cortical neurons obtained from WT, ncx3-/- and siah2-/- mice. In particular, the choice of siah2-/- mouse model is due to the finding that during hypoxia AKAP121 undergoes to proteolytic degradation mediated by the activation of the E3 ubiquitin-ligase Siah2 (Carlucci et al., 2008). Therefore, this model represents a condition in which AKAP121 is hyperexpressed during hypoxia. Hypoxia was reproduced *in vitro* by incubating neurons in a medium deprived of oxygen and glucose for 3 hr (OGD) followed by 24 hr reoxygenation (Reoxy). In the first step of the study, NCX3 expression was evaluated in siah2+/+ and siah2-/- neurons exposed to OGD and OGD/Reoxy. Interestingly, the results obtained demonstrated that NCX3 expression was reduced in siah2+/+ neurons exposed to hypoxia and returns to the basal levels during the reoxygenation phase. Conversely, NCX3 expression did not change neither in OGD nor in OGD/Reoxy in siah2-/- cells. This finding suggests that, in siah2-/- neurons, besides AKAP121, NCX3 is not degraded during hypoxia.

Then, the study moved to the evaluation of mitochondrial dynamics in siah2+/+ and siah2-/- neurons in both hypoxic and reoxygenation conditions, by using a biochemical and a morphological approach. To this aim Western Blot analysis was performed to measure the expression levels of Drp1 and Mfn1, two proteins considered markers of mitochondrial fission and fusion, respectively. The results obtained showed a reduction of Mfn1 and a raise in Drp1 expression in siah2+/+ neurons exposed to OGD, whereas in siah2-/- neurons no changes in the expression of these two proteins were observed during OGD compared to their levels measured in normoxia. The exposure to OGD/Reoxy induced a raise in Mfn1 expression in both siah2+/+ and siah2-/- neurons, whereas Drp1 levels increased in siah2+/+ neurons and resulted unchanged in siah2-/- neurons compared to its expression in basal conditions. Collectively, these results suggests that mitochondrial fission prevails during hypoxic conditions in siah2+/+ neurons, whereas mitochondrial fusion is predominant in siah2+/+ neurons exposed to OGD/Reoxy. Interestingly, in siah2-/- neurons the balance between fusion and fission in both hypoxia and reoxygenation is shifted toward fusion. This results were confirmed by the morphological analysis in living neurons stained with the fluorescent probe MitoTracker Red. Indeed, confocal microscopy showed that mitochondria underwent fragmentation upon hypoxia in siah2+/+ neurons, as demonstrated by the reduction of those parameters that measure the length and the branching of mitochondria, such as aspect ratio (AR) and form factor (FF) respectively, whereas these parameters did not change in siah2-/- neurons. Moreover, in siah2+/+ neurons exposed to OGD/Reoxy, AR and FF levels were similar to those measured in the same neurons in basal conditions, indicating that mitochondrial morphology was restored during the phase of reoxygenation. Interestingly, in siah2-/- neurons exposed to OGD followed by reoxygenation both AR and FF were significantly higher than their levels

observed in normoxic and hypoxic conditions, indicating that mitochondria were much longer and interconnected in the reoxygenation phase.

The next step of the study was addressed to evaluate the mitochondrial function in siah2+/+ and siah2-/- neurons during hypoxia and reoxygenation. To this aim mitochondrial calcium concentration, $[Ca^{2+}]_m$, and mitochondrial membrane potential, $\Delta\Psi_m$, were evaluated by staining the cell with the fluorescent probes XRHOD1 and TMRE, respectively, and using the confocal microscopy. The results obtained in siah2+/+ neurons showed that OGD and OGD followed by reoxygenation strongly increased $[Ca^{2+}]_m$ and reduced $\Delta\Psi_m$. Conversely, the levels of $[Ca^{2+}]_m$ measured in siah2-/- neurons exposed to OGD and OGD/Reoxy were significantly lower than those observed in siah2+/+ neurons in the same conditions. In addition, the exposure to of siah2-/- neurons to OGD did not change $\Delta\Psi_m$ compared to basal conditions, while an hyperpolarization of mitochondrial membrane was detected during reoxygenation. These results let to hypothesize that in siah2-/- neurons exposed to OGD, the lack of AKAP121and NCX3 ubiquitination due to hypoxia-induced Siah2 activation, prevents mitochondrial Ca^{2+} uptake and mitochondrial membrane depolarization observed in siah2+/+ neurons exposed to hypoxia and reoxygenation. These hypothesis was supported by the experiments assessing the mitochondrial oxidative capacity. Indeed, mitochondrial oxidative activity was impaired in siah2+/+ neurons exposed to OGD and to OGD/Reoxy, whereas it was significantly improved in siah2-/- neurons in the above mentioned experimental conditions. Accordingly, ATP production was reduced in siah2+/+ neurons exposed to hypoxia, whereas it was not modified in siah2-/- neurons exposed to OGD compared to control conditions.

Likewise to the analysis conducted in siah2-/- model, further experiments were performed in neurons obtained from ncx3-/- mice in order to confirm the role played by NCX3 in the regulation of the mitochondrial dynamics during hypoxia. Previous data obtained in our laboratories demonstrated that, in basal conditions mitochondria in ncx3-/- neurons looks fragmented compared to mitochondria observed in ncx3+/+ neurons. Surprisingly, this evidence did not correlate with an alteration in the expression of Drp1 and Mfn1 that were similar in ncx3+/+ and in ncx3-/- neurons. However, the exposure of neurons to OGD and OGD/reoxygenation induced a reduction in Mfn1 expression during hypoxia that returned to the basal values during the reoxygenation phase, whereas the expression of Drp1 was not affected by OGD or OGD/Reoxy, neither in ncx3+/+ nor in ncx3-/- neurons, thus confirming that the lack of NCX3 during hypoxia plays a key role in the balance between mitochondrial fission and fusion during hypoxia and reoxygenation. Indeed from a functional point of view, a strong reduction of ATP levels was induced by hypoxia followed by reoxygenation in both ncx3+/+ and ncx3-/- neurons exposed to OGD/Reoxy, whereas $[Ca^{2+}]_m$ measured in ncx3+/+ and ncx3-/- neuronal cells was higher in ncx3-/- compared to ncx3+/+ neurons already in basal conditions.

Collectively, these findings suggest that the interaction between NCX3 and AKAP121 has a crucial role in the regulation of mitochondrial dynamic due to its ability to promote mitochondrial Ca^{2+} homeostasis during hypoxia and reoxygenation.

These findings lead to the identification of new molecular targets useful to hypothesize alternative strategies for the treatment of brain ischemia.

3. Experimental procedures

Cell Culture

Primary cortical neurons from ncx3-/- and siah2-/- mice were obtained from 15-16 days old embryos as previously reported (Molinaro et al., 2008). Brains were removed, cortices isolated and subjected to mechanical and enzymatic digestion for 30 minutes in presence of trypsin/EDTA at 37°C. After incubation, the tissues were centrifuged (2500 RPM, 5 minutes), the supernatant was removed and the pellet was resuspended in MEM/F12 culture medium (Life technologies) containing: Horse Serum (5%), Fetal Bovine Serum (5%), Glucose 30% (0,6%) and antibiotics (penicillin/streptomicyn 0,5%). Neurons were then plated in plastic petri-dishes, for Western Blot experiments, or on 25mm glass cover slip for confocal experiments. Neurons were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and used after 8 days.

Combined oxygen and glucose deprivation (OGD) and reoxygenation (Reoxy)

Cortical neurons were exposed to OGD for 3 hrs followed by 24 hrs reoxygenation according to a previously reported protocol (Goldberg et al., 1993). Briefly, the culture medium was replaced with a hypoxia medium previously saturated for 20 min with 95% N_2 and 5% CO_2 and containing NaCl 116 mM, KCl 5.4 mM, $MgSO_4$ 0.8 mM, $NaHCO_3$ 26.2 mM, NaH_2PO_4 1 mM, $CaCl_2$ 1.8 mM, glycine 0.01 mM and 0.001 w/v phenol red.

Hypoxic conditions were maintained using a hypoxia chamber (temperature 37°C, atmosphere 95% N_2 and 5% CO_2). These experimental conditions induced 30% decrease of pO_2 in the medium.

Deprivation of oxygen and glucose was stopped by placing the cells in the regular culture medium saturated with a mixture of 95% O_2 and 5% CO_2 for 10 min. Reoxygenation was achieved by returning neurons to normoxic conditions (37°C in a humidified 5% CO_2 atmosphere) for 24 hrs.

Analysis of mitochondrial morphology using the IMAGEJ 1.42 software

To label mitochondria, the cells were incubate with MitoTracker Red (Invitrogen, 20 nM) for 15 min prior to fixation. Digital images were captured on a confocal microscope, using a 63 X oil immersion lens and subjected to a 2D deconvolution step, which is meant to compensate for optical imperfections of the microscope. To this end, we used the ‘‘Interative Deconvolution’’, a plug-in written by Bob Dougherty for ImageJ. After these image enhancements, mitochondrial shape metrics were reported by an ImageJ macro, "Morphometry", described by Cribbs and Strack (Cribbs and Strack, 2009). This macro allowed us to determine two parameters of mitochondrial morphology: form factor (FF) and aspect ratio (AR). The aspect ratio (major axis divided by minor axis) is a useful shape metric for simple rod-like mitochondria, but it does not faithfully represent the shape of kinked, branched, or highly interconnected mitochondria. The form factor takes into account perimeter and area and can therefore capture complex mitochondrial shapes. As the inverse of circularity, form factors range from 1 for a perfect circle to infinity as the

ratio of particle perimeter to area increases. Low values of FF and AR are index of circular mitochondria, high values indicate that mitochondria are elongated and highly interconnected.

Western Blot

Protein samples (50 µg) were analyzed on 8% (NCX3) or 10% (Drp1 and Mfn1) sodium dodecyl sulfate polyacrilamide gel with 5% sodium dodecyl sulfate stacking gel (SDS-PAGE) and electrotransferred onto Hybond ECL nitrocellulose paper (Amersham). Membranes were blocked with 5% non fat dry milk in 0.1% Tween-20 (TBS-T; 2 mM Tris–HCl, 50 mM NaCl, pH 7.5) for 2 h at RT and subsequently incubated overnight at 4°C in the blocked buffer with the antibodies for NCX3 (polyclonal rabbit antibody, Philipson's Laboratory; 1:5000), Mfn1 (Millipore, 1:1000), Drp1 (BD biosciences, 1:1000), Tubulin (Sigma-Aldrich, 1:10000), Actin (Sigma-Aldrich, 1:1000). The membranes were washed with 0.1% Tween 20 and incubated with the secondary antibodies for 1 h (1:1000; Amersham). Immuno-reactive bands were detected with the ECL (Amersham).

Imaging mitochondrial Ca^{2+} and mitochondrial membrane potential

$[Ca^{2+}]_m$ was assessed using the fluorescent dye X-Rhod1. Cells were loaded with X-Rhod1 0.2 µM for 15 min in a medium containing 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25

mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes. The pH was adjusted to 7.35 with NaOH. At the end of the incubation, cells were washed 3 times in the same medium. An increase in mitochondria-localized intensity of fluorescence was indicative of mitochondrial Ca^{2+} overload.

Mitochondrial membrane potential was assessed using the fluorescent dye tetramethyl rhodamine ethyl ester (TMRE) in the "redistribution mode". Cells were loaded with TMRE (20 nM) for 30 min in the above described medium. At the end of the incubation, the cells were washed in the same medium containing TMRE (20 nM) and allowed to equilibrate. A decline in mitochondria-localized intensity of fluorescence was indicative of mitochondrial membrane depolarization.

Confocal images were obtained using a Zeiss inverted 700 confocal laser scanning microscopy and a 63X oil immersion objective. The illumination intensity of 543 Xenon laser, used to excite X-Rhod-1 and TMRE fluorescence, was kept to a minimum of 0.5% of laser output to avoid phototoxicity.

ATP detection

ATP content was measured with a commercial bioluminescent assay (ATP bioluminescent assay kit, Sigma, St. Louis, Missouri, USA) according to manufacturer's instruction. Briefly, ATP was extracted by boiling the samples in a solution containing (in mM) 100 TRIS, 4 EDTA, pH 7.75, and then centrifuged at 10,000×g for 60 s**.** To obtain bioluminescence measurements with a standard luminometer, 100µL of supernatant was mixed with 100 µL of luciferin–luciferase solution. The standard curve of ATP was

obtained by serial dilution of 2μM ATP solution (Maeda 2003).

MTT assay

Mitochondrial activity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT) assay as previously described (Sisalli et al., 2014). The assay was based on the red-ox ability of living mitochondria to convert dissolved MTT into insoluble formazano. Briefly, after treatments, the medium was removed and cells were incubated in 1ml of MTT solution (0.5 mg/ ml) for 1 h in a humidified 5% CO_2 incubator at 37°C. To stop incubation, MTT solution was removed and 1ml dimethyl sulfoxide was added to solubilize the formazan produced. The absorbance was monitored at 540 nm with a Perkin-Elmer LS 55 luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, England). The data are expressed as percentage of cell damage compared with sham-treated cultures.

Statistical Analysis

Data were generated from a minimum of three independent experiments. Calcium measurements were performed at least in 20 cells for each of the three independent experimental sessions. Data are expressed as mean ± S.E.M. Statistical analysis was performed with one way variance analysis (ANOVA) followed by Newman-Keuls test. Statistical significance was accepted at the 95% confidence level ($P \pm 0.05$).

4. RESULTS

4.1. Effect of OGD and OGD/Reoxygenation on NCX3 expression in primary cortical neurons from siah2+/+ and siah2-/- mice.

In the first step of the study, NCX3 expression was evaluated in siah2+/+ and siah2-/- neurons exposed to OGD and OGD/Reoxygenation to understand whether there is a relationship between NCX3 degradation and Siah2 activation during hypoxia. The results obtained demonstrated that NCX3 expression was reduced in siah2+/+ neurons exposed to hypoxia and returned to the basal levels during the reoxygenation phase. Conversely, NCX3 expression did not change in siah2-/- cells after the exposure to OGD and OGD followed by Reoxygenation (Fig. 5).

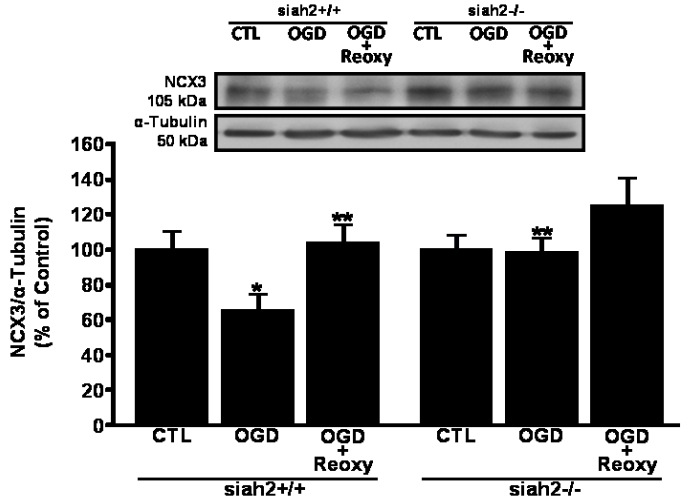

Fig. 5. Effect of OGD and OGD/Reoxygenation on NCX3 expression in siah2+/+ and siah2-/- neurons. Western Blotting analysis of NCX3 protein expression in primary cortical neurons from siah2+/+ and siah2-/- mice exposed to 3h of OGD and OGD followed by 24h of reoxygenation. Each bar represents the mean±s.e.m. of the percentage of different experimental values obtained in three independent experimental sessions. *P<0.05 vs siah2+/+ CTL; **P<0.05 vs siah2+/+ OGD.

This finding suggests that in siah2-/- neurons, besides AKAP121 (Carlucci et al., 2008, NCX3 is not degraded during hypoxia.

4.2. Effect of OGD and OGD/Reoxygenation on mitochondrial morphology in primary cortical neurons from siah2+/+ and siah2-/- mice

Once demonstrated that NCX3 did not undergo to proteolytic degradation during OGD in siah2-/- mice, further experiments were performed in this model to evaluate the role played by NCX3 and AKAP121 in the regulation of mitochondrial morphology. To this aim Western Blotting experiments were conducted in siah2-/- neurons exposed to OGD and OGD/Reoxygenation to evaluate Drp1 and Mfn1 expression, two markers of mitochondrial fission and fusion events, respectively. This analysis showed that the exposure of siah2+/+ neurons to OGD induced an increase in Drp1 expression and a reduction in Mfn1 (Fig. 6, A and B), whereas, in siah2-/- neurons exposed to OGD no changes in Drp1 and Mfn1 expression occurred (Fig. 6, A and B). Finally, the exposure to OGD/Reoxygenation significantly increased Mfn1 protein levels in both siah2+/+ and siah2-/- neurons while did not affect Drp1 protein expression (Fig. 6, A and B).

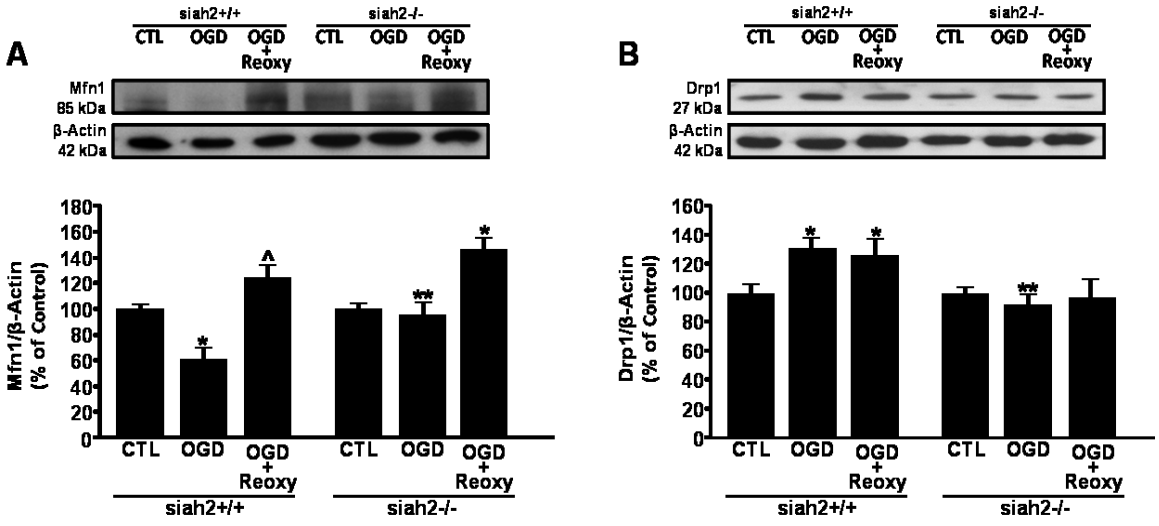

Fig. 6. Effect of OGD and OGD/Reoxygenation on the expression of proteins regulating mitochondrial fusion and fission, n in siah2+/+ and siah2-/- neurons. Western Blotting analysis of **(A)** Mfn1 and **(B)** Drp1 expression in primary cortical neurons obtained from siah2+/+ and siah2-/- mice and exposed to 3h of OGD and OGD followed by 24h of reoxygenation. Each bar represents the mean±s.e.m. of the percentage of different experimental values obtained in three independent experimental sessions. **P*<0.05 vs siah2+/+ CTL or siah2-/- CTL; ***P*<0.05 vs siah2+/+ OGD; ^*P*<0.05 vs siah2+/+ CTL and siah2+/+ OGD.

These findings were supported by parallel experiments aimed to evaluate mitochondrial morphology by confocal microscopy approach. This analysis allows to measure the FF and the AR, two parameters which respectively indicate the degree of branching and the length of mitochondria, in neurons stained with the fluorescent probe MitoTracker Red. The results reported in Fig. 7 demonstrated that the exposure to OGD caused a reduction of FF and AR in siah2+/+ neurons, whereas it did not affect these parameters in siah2-/-neurons. During the reoxygenation phase FF and AR were restored in siah2+/+ neurons, whereas were significantly increased in siah2-/- neurons compared to the levels observed in control neurons (Fig. 7, A and B).

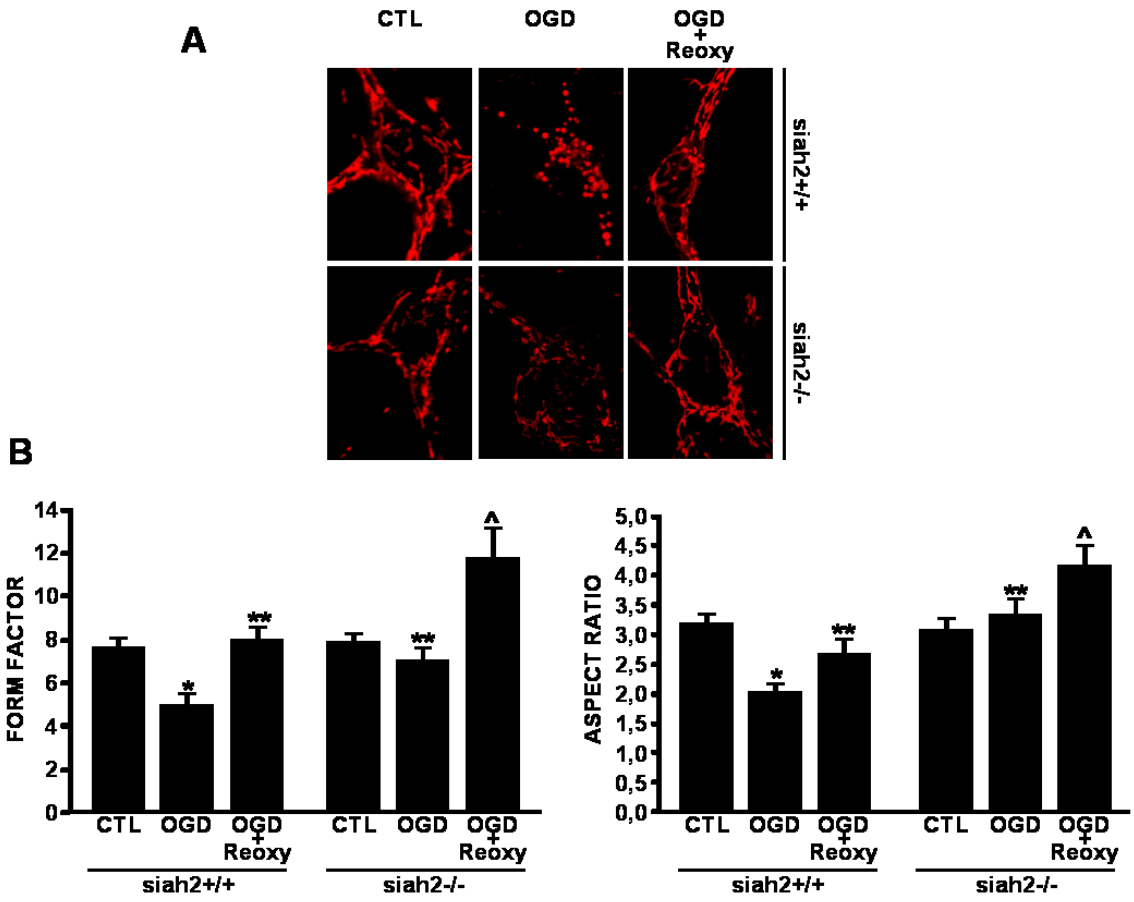

Fig. 7. Effect of OGD and OGD/Reoxygenation on mitochondrial morphology in siah2+/+ and siah2-/- neurons. (A) Representative cortical neurons exposed to 3h of OGD and OGD followed by 24h of reoxygenation and stained with the fluorescent probe MitoTracker Red. **(B)** Quantification of Form Factor (FF) and Aspect Ratio (AR) parameters in the above mentioned neurons. Each bar represents the mean±s.e.m. of at least 15–20 neurons recorded in three independent experimental sessions. **P*<0.05 vs siah2+/+ CTL; ***P*<0.05 vs siah2+/+ OGD; ^*P*<0.05 vs siah2+/+ OGD/Reoxy and siah2-/- OGD.

Collectively, the results obtained let to hypothesize that the lack of proteolysis of NCX3 and AKAP121 occurring in neurons missing Siah2, preserves mitochondrial morphology during OGD as compared to siah2+/+ neurons.

4.3. Effect of OGD and OGD/Reoxygenation on mitochondrial functional properties in primary cortical neurons from siah2+/+ and siah2-/- mice

In order to correlate the changes in mitochondrial morphology occurring during OGD to the alterations in the functional state of mitochondria, mitochondrial parameters were compared in siah2+/+ and siah2-/- neurons exposed to OGD and OGD/Reoxygenation.

To this aim mitochondrial calcium concentration, $[Ca^{2+}]_m$, and mitochondrial membrane potential, $\Delta\Psi_m$, were evaluated in siah2+/+ and siah2-/- neurons by staining the cells with the fluorescent probes XRHOD1 and TMRE, respectively, and using the confocal microscopy. The results obtained demonstrated that $[Ca^{2+}]_m$ was increased after the exposure of siah2+/+ neurons to OGD and OGD/Reoxygenation, whereas $\Delta\psi_m$ was reduced (Fig. 8, A and B). Interestingly, $\Delta\psi_m$ was not altered in siah2-/- subjected to OGD, while during the reoxygenation phase an hyperpolarization of mitochondrial membrane occurred (Fig. 8A). In addition, during the exposure to both OGD and OGD/Reoxygenation the levels of $[Ca^{2+}]_m$ were significantly lower in siah2-/- neurons than those observed in siah2+/+ neurons (Fig. 8B).

Then, mitochondrial red-ox activity was assessed in order to better understand the functional state of mitochondria in siah2-/- neurons exposed to OGD and reoxygenation.

These experiments demonstrated that in siah2+/+ neurons the exposure to OGD and OGD/Reoxygenation led to an impairment of mitochondrial oxidative activity that was significantly improved in siah2-/- neurons exposed to the same experimental conditions (Fig. 8C).

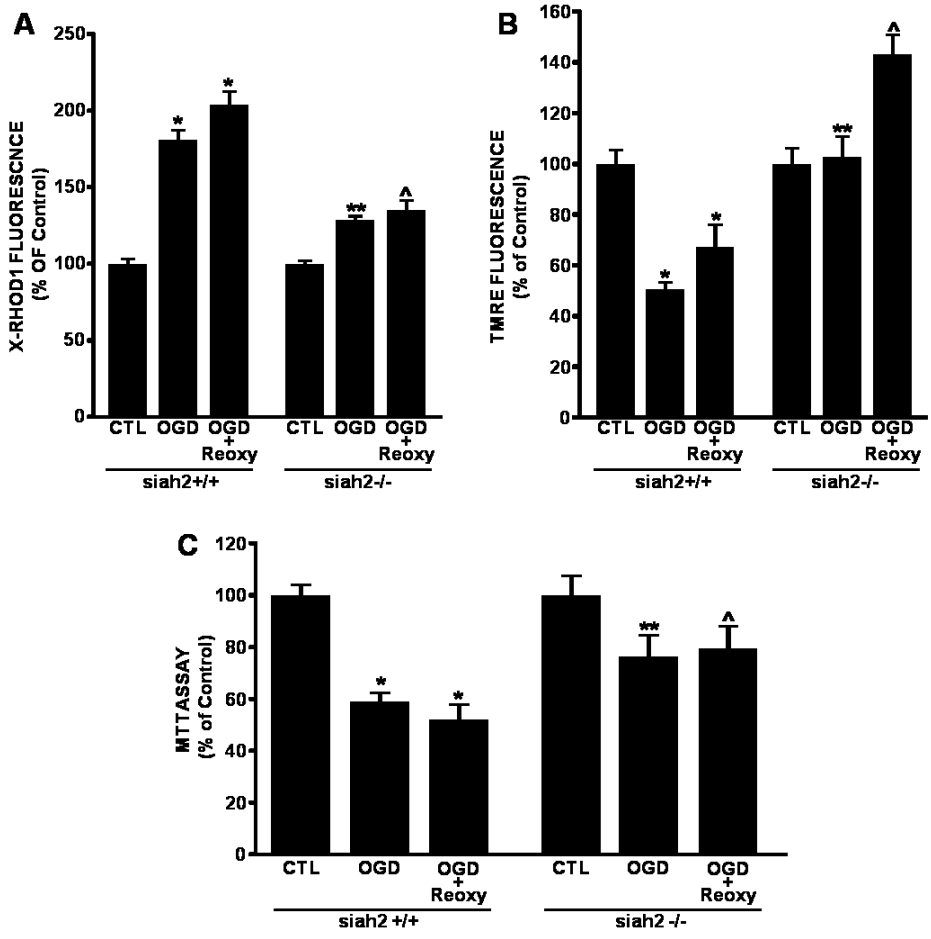

Fig. 8. Effect of OGD and OGD/Reoxygenation on mitochondrial functional properties in siah2+/+ and siah2-/- neurons. Determination of **(A)** $[Ca^{2+}]_m$, **(B)** $\Delta\psi_m$ and **(C)** mitochondrial oxidative capacity in primary cortical neurons obtained from siah2+/+ and siah2-/- mice and exposed to 3h of OGD and OGD followed by 24h of reoxygenation. Each bar represents the mean±s.e.m. of the percentage values of at least 20–30 neurons recorded in three independent experimental sessions. **P*<0.05 vs siah2+/+ CTL; ***P*<0.05 vs siah2+/+ OGD; ^*P*<0.05 vs siah2+/+ OGD/Reoxy.

These results are consistent with those obtained for $[Ca^{2+}]_m$ and $\Delta\psi_m$ and confirmed that the lacking of AKAP121 and NCX3 degradation in siah2-/- neurons leads to a significant improvement of the mitochondrial function during OGD and OGD/Reoxygenation.

Furthermore, ATP measurement confirmed these findings. Indeed, the exposure to OGD induced an impairment of ATP generation in siah2+/+ neurons, whereas any change was detected in siah2-/- (Fig. 9), confirming that in siah2-/- neurons the mitochondrial function was significantly ameliorated compared to siah2+/+ neurons.

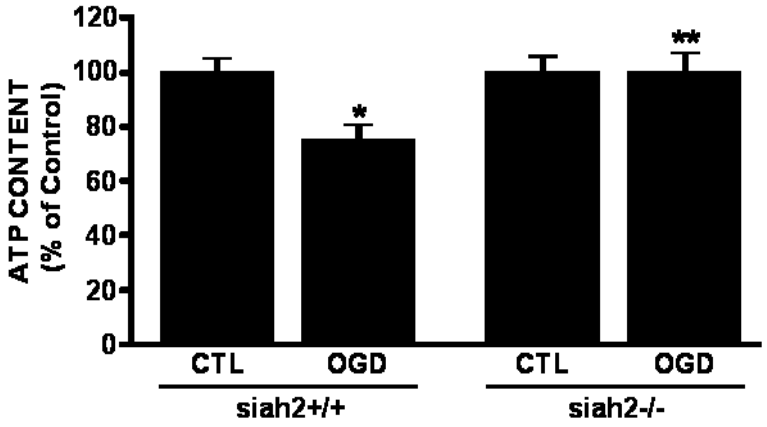

Fig. 9. Effect of OGD on ATP production in siah2+/+ and siah2-/- neurons. Quantification of ATP production in primary cortical neurons from siah2+/+ and siah2-/- mice exposed to 3h of OGD. Each bar represents the mean±s.e.m. of the percentage of different experimental values obtained in three independent experimental sessions. **P*<0.05 vs siah2+/+ CTL; ***P*<0.05 vs siah2+/+ OGD.

4.4. Effect of OGD and OGD/Reoxygenation on Drp1 and Mfn1 expression in primary cortical neurons from ncx3+/+ and ncx3-/- mice

Likewise to the analysis conducted in siah2-/- model, further experiments were performed in neurons obtained from ncx3-/- mice in order to confirm the role played by NCX3 in the regulation of the mitochondrial dynamics during hypoxia. Since previous data obtained in our laboratories demonstrated that in ncx3-/- neurons mitochondria display a fragmented shape in basal conditions, Drp1 and Mfn1 protein expression was assessed in ncx3+/+ and ncx3-/- neurons. The results reported in Fig. 10 showed that no changes in Drp1 and Mfn1expression were detected ncx3-/- neurons in comparison with ncx3+/+ cells (Fig. 10, A and B). Interestingly, the exposure of ncx3-/- neurons to OGD and OGD/Reoxy, two conditions that modulate NCX3 expression (Sirabella et al., 2009), did not affect Drp1 expression (Fig. 10A), whereas it influenced Mfn1 expression. Indeed, Mfn1 was reduced in ncx3-/- neurons during OGD and returned to the basal level in the reoxygenation phase (Fig. 10B). However, no differences in the expression of Mfn1 and Drp1 were detected between ncx3+/+ and ncx3-/- neurons exposed to OGD and OGD/Reoxy (Fig. 10, A and B) thus confirming that the lack of NCX3 during hypoxia plays a key role in the balance between mitochondrial fission and fusion during hypoxia and reoxygenation.

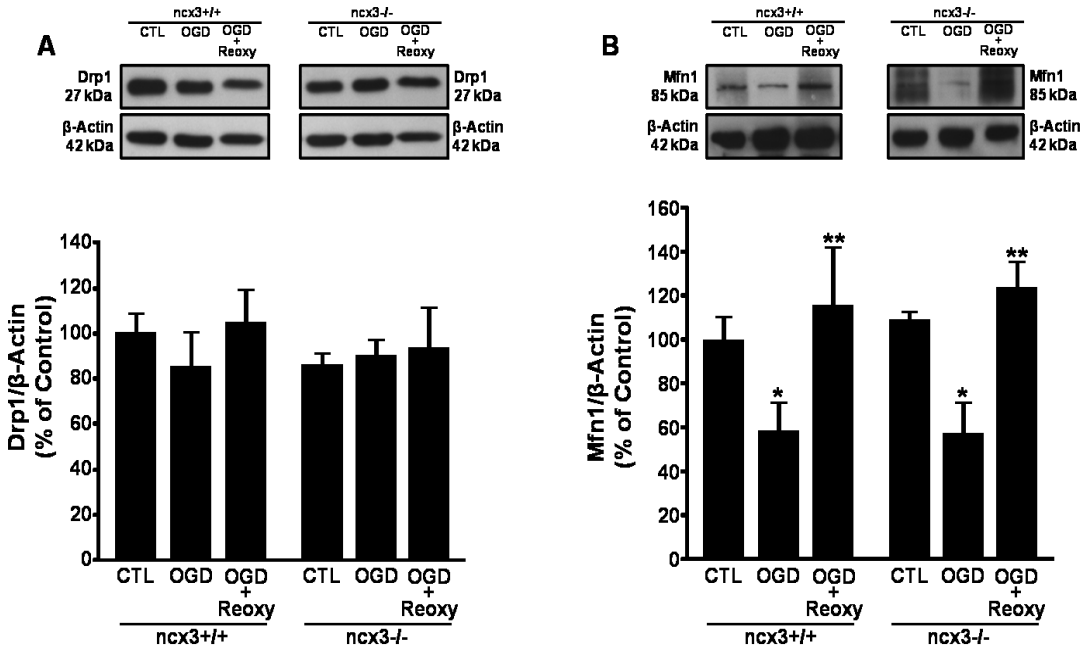

Fig. 10. Effect of OGD and OGD/reoxygenation on the expression of proteins regulating mitochondrial fission and fusion in ncx3+/+ and ncx3-/- neurons. Western Blotting analysis of **(A)** Drp1 and **(B)** Mfn1 expression in primary cortical neurons from ncx3+/+ and ncx3-/- mice exposed to 3h of OGD and OGD followed by 24h of reoxygenation. Each bar represents the mean±s.e.m. of the percentage of different experimental values obtained in three independent experimental sessions. **P*<0.05 vs ncx3+/+ CTL or ncx3-/- CTL; ***P*<0.05 vs ncx3+/+ OGD and ncx3-/- OGD.

4.5. Analysis of the mitochondrial function in primary cortical neurons from ncx3+/+ and ncx3-/- mice exposed to OGD/Reoxygenation

The study of Drp1 and Mfn1 expression in ncx3-/- neurons was paralleled by the evaluation of mitochondrial function parameters such as ATP production and $[Ca^{2+}]_m$. The results obtained showed that ATP generation was strongly impaired in ncx3+/+ and ncx3-/-

neurons after the exposure to OGD followed by Reoxygenation (Fig. 11A). However, any difference in ATP production was detected between ncx3+/+ and ncx3-/- neurons in both basal conditions and after OGD followed by reoxygenation. As expected, $[Ca^{2+}]_m$ was higher in ncx3-/- neurons compared to ncx3+/+ neurons already in basal condition (Fig. 11B), indicating that NCX3 plays an active role in the regulation of mitochondrial Ca^{2+} homeostasis.

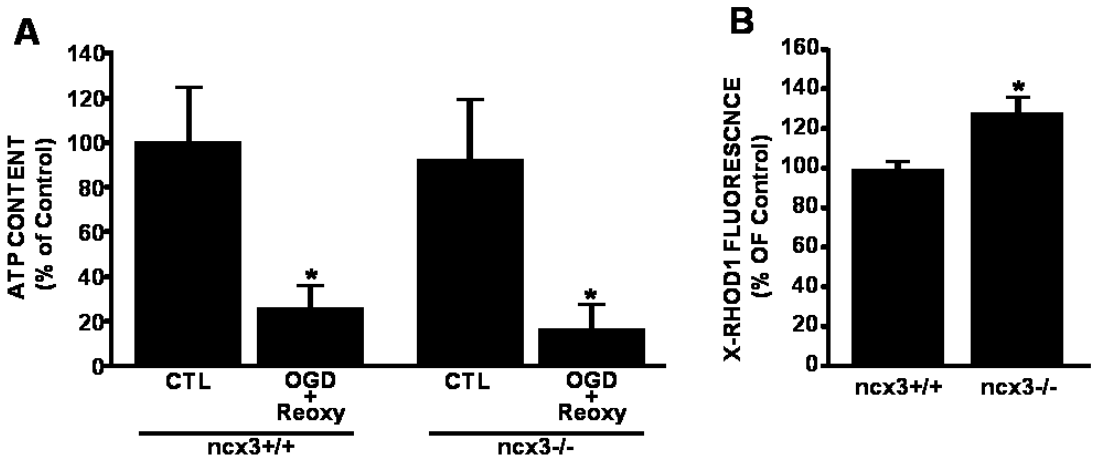

Fig. 11. Mitochondrial functional properties in ncx3+/+ and ncx3-/- neurons. (A) Quantification of ATP production in primary cortical neurons from ncx3+/+ and ncx3-/- mice exposed to OGD followed by 24h of reoxygenation. Each bar represents the mean±s.e.m. of the percentage of different experimental values obtained in three independent experimental sessions**. (B)** Measurement of $[Ca^{2+}]_m$ (as percentage of control) in ncx3+/+ and ncx3-/- neurons in basal conditions. Each bar represents the mean±s.e.m. of at least 20–30 neurons recorded in three independent experimental sessions. **P*<0.05 vs ncx3+/+ CTL or ncx3-/- CTL

5. DISCUSSION

Despite the functional link between oxygen availability and mitochondrial function, the possible relationship between mitochondrial fission and fusion events and mitochondrial adaptations to hypoxia is largely unexplored. This study demonstrate that hypoxia triggers mitochondrial fragmentation paralleled by an alteration in the expression levels of Drp1 and Mfn1, crucial proteins involved in the regulation of mitochondrial fission and fusion events, respectively.

Moreover, the results of the present study also demonstrate that the interaction between NCX3 and AKAP121, two mitochondrial proteins involved in the regulation of mitochondrial metabolism and mitochondrial calcium homeostasis in physiological and pathological conditions (Livigni et al., 2006; Scorziello et al., 2013; Sisalli et al., 2014), allows the maintenance of mitochondrial morphology during hypoxia and reoxygenation by preventing the overload of Ca^{2+} into mitochondria. Indeed, both the biochemical and the morphological analysis performed in wild type neurons exposed to OGD, an experimental condition leading to ubiquitin-induced NCX3 and AKAP121 proteolytic degradation (Sirabella et al., 2009; Carlucci et al., 2008), are suggestive of mitochondrial fragmentation as testified by the increase in Drp1 protein expression associated to a reduction in the levels of Mfn1. Furthermore, in the same experimental conditions FF and AR, two parameters used to measure mitochondrial morphology, assumed values indicative of a shift from fused to fragmented mitochondria. From a functional point of view, the changes in mitochondrial morphology described in OGD correlate with a mitochondrial depolarization and a massive increase in $[Ca^{2+}]_m$, thus suggesting that mitochondrial dysfunction strictly correlates with alterations in mitochondrial dynamics (Twig et al.,

2008; Legros et al., 2002, Duvezin-Caubet et al., 2006). Therefore, it is likely that in the above mentioned experimental condition mitochondrial fragmentation occurs to face the metabolic injury arising in hypoxic conditions. Indeed, in these cells ATP production and mitochondrial redox activity were strongly impaired. This is in line with data recently reported by Shutt and McBride (Shutt and McBride, 2013) showing that depolarized mitochondria undergo to fragmentation, and that this event leads to the clearance of these organelles through the autophagic pathway, thus indicating that mitochondrial dynamics might be part of a more complex mechanism aimed to allow mitochondrial quality control. Conversely, in siah2-/- neurons exposed to OGD no changes in mitochondrial morphology, Drp1 and Mfn1 protein expression have been detected. More importantly, in these neurons mitochondrial function was preserved as confirmed by the levels of $[Ca^{2+}]_m$, that were lower compared to siah2+/+ neurons, as well as mitochondrial membrane that results hyperpolarized in comparison to that observed in siah2+/+ neurons exposed to OGD. Similarly, ATP production and mitochondrial redox activity are preserved in siah2-/- neurons compared to siah2+/+ cells exposed to OGD. Interestingly, in these cells the expression of NCX3 and AKAP121 was not affected by OGD exposure, thus confirming the role played by the hypoxia-induced E3 ubiquitin-ligase Siah2 in the regulation of AKAP121 and NCX3 expression during OGD (Carlucci et al. 2008, present paper). Moreover, these finding also support the hypothesis that the presence of AKAP121 and NCX3 on the outer mitochondrial membrane is crucial not only to preserve mitochondrial function in metabolic stress conditions such as hypoxia, but also for the maintenance of mitochondrial morphology. According with data previously reported (Carlucci et al., 2008; Narendra et al., 2010; Vivez-Bauza et al., 2010; Jin et al., 2010; Matsuda et al., 2010; Ziviani et al., 2010) we hypothesize that Siah2 activated by hypoxia is recruited by

depolarized mitochondria where it induces the degradation of the outer membrane proteins, NCX3 and AKAP121, via proteasome as it occurs for the complex Pink1/parkin in Parkinson's disease (Tanaka et al., 2010; Chan and Salazar, 2011).

Another aspect of the study that deserve to be underlined is that the lack of degradation of NCX3 and AKAP121 observed in siah2-/- neurons during OGD plays a neuro-beneficial effect also during the reoxygenation phase. Indeed, in the reoxygenation phase, in siah2-/- neurons $[Ca^{2+}]_m$ was lower compared to siah2+/+ cells, their redox activity is greatly improved and mitochondrial membrane hyperpolarized. From a morphological point of view, these findings correlate with mitochondria much more elongated and interconnected compared to those observed in basal conditions. Conversely, in siah2+/+ neurons exposed to OGD followed by reoxygenation, although Mfn1 protein expression returns to values comparable to those observed in basal conditions, and FF and AR reach values suggestive of mitochondrial fusion, the functional proprieties of these organelles are worsened. Indeed, mitochondrial membrane is depolarized, $[Ca^{2+}]_m$ is higher and the redox activity is impaired as well as in neurons exposed to OGD alone. These findings are in line with data previously reported showing that overly fused mitochondria may accumulate oxidative stress (Itoh et al., 2013). Moreover, it is well known that the balance between mitochondrial fusion proteins, such as Mfn1, Mfn2 and Opa1, and fission proteins including Drp1, is an important requirement to assure neuronal survival (Itoh et al., 2013, Bertholet et al., 2015, Bhat et al., 2015, Burtè et al., 2015; Zorzano and Claret, 2015), since many human diseases are associated with changes in mitochondrial function and morphology (Chan 2012). Therefore, it is possible to speculate that in our experimental conditions the lack of interaction between NCX3 and AKAP121 at mitochondrial level during OGD is responsible for the detrimental effects we observed in the reoxygenation

phase on mitochondrial metabolism which, in turn, leads to an imbalance in mitochondrial dynamics. This hypothesis is strongly supported by the data obtained in siah2-/- neurons in which NCX3 and AKAP121 protein expression is not affected by OGD and mitochondrial function and morphology are preserved also in the subsequent reoxygenation phase. Together, these findings confirm that the interaction between NCX3 and AKAP121 on the outer mitochondrial membrane plays a crucial role in the regulation of mitochondrial morphology in hypoxia/reoxygenation, and it links the changes in mitochondrial morphology with metabolic cell conditions. On the other hand, AKAP121 also acts as a regulator of mitochondrial morphology by modulating Drp1 activity and recycling. Indeed, AKAP121 induces Drp1 inhibition through its phosphorylation in a PKA dependent manner, and it hampers Drp1-Fis1 interaction on OMM (Kim et al., 2011; Merrill et al., 2011).

Furthermore, the experiments performed in ncx3-/- neurons provide new evidence supporting the role of AKAP121 in the regulation of mitochondrial dynamics since they, demonstrate that NCX3 is a key element of the complex with AKAP121, and the interaction between these two protein is necessary to maintain mitochondrial morphology. Indeed, in basal conditions in ncx3-/- neurons mitochondria look fragmented compared to mitochondria observed in ncx3+/+ neurons (unpublished results). Surprisingly, this evidence did not correlate with an alteration in the expression of Drp1 and Mfn1 that were similar in ncx3+/+ and in ncx3-/- neurons. However, the exposure of neurons to OGD and OGD/reoxygenation induced a reduction in Mfn1 expression during hypoxia that returned to the basal values during the reoxygenation phase, whereas the expression of Drp1 was not affected by OGD or OGD/reoxygenation, neither in ncx3+/+ nor in ncx3-/- neurons. These findings confirm that the lack of NCX3 during hypoxia importantly affects the

balance between mitochondrial fission and fusion during hypoxia and reoxygenation. Indeed, from a functional point of view, a strongly reduction of ATP levels was induced by hypoxia followed by reoxygenation in both ncx3+/+ and ncx3-/- neurons, whereas $[Ca^{2+}]_m$ was higher in ncx3-/- compared to ncx3+/+ cells, already in basal condition, thus confirming the main role played by NCX3 in the interaction with AKAP121 as crucial regulator of mitochondrial dynamics. In fact, NCX3, owing to its ability to promote mitochondrial Ca^{2+} homeostasis during hypoxia and reoxygenation, preserves mitochondrial function and in turn mitochondrial morphology.

These findings allow to conclude that NCX3 controlling mitochondrial morphology, mitochondrial oxidative metabolism and neuronal survival might be included among the new regulators of mitochondrial dynamics in hypoxia/reoxygenation. Moreover, these results also demonstrate that AKAP121 works as an ancillary element in the complex with NCX3, since in the absence of the exchanger AKAP121 is unable to preserve the functions of mitochondria and consequently their morphology.

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