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"MOLECULAR MECHANISMS INVOLVED IN MITOCHONDRIAL CALCIUM HOMEOSTASIS AND THEIR RELATIONSHIP WITH MITOCHONDRIAL DYNAMICS IN IN VITRO MODELS OF CEREBRAL ISCHEMIA"

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1.INTRODUCTION

1.1 Background

Mitochondria are highly specialized organelles and major players in fundamental aspects of cell physiology. In eukaryotic cells, energy production is functionally coupled to metabolic demands and the cells efficiently adapt oxidative respiration in response to changes in extracellular microenvironment and metabolic nutrient availability.

These organelles produce approximately 15 times more ATP from glucose than the glycolytic pathway in eukaryotic cells by coupling electron transport to the generation of proton gradients for oxidative phosphorylation. Cells of highly metabolic tissues such as muscle, liver and brain, are therefore particularly dependent on mitochondria. In particular, in the central nervous system mitochondria produce over 95% of ATP utilized by the brain (Erecinska and Silver, 1994) and within neurons they are distributed to regions of high metabolic demand, including synapses, nodes of Ranvier and myelination/ demyelination interfaces (Berthold et al., 1993; Bristow et al., 2002; Kageyama and Wong-Riley, 1982; Rowland et al., 2000). The generation, processing and transmission of neural impulses rely heavily on sodium (Na^+), potassium (K^+) and calcium

(Ca^{2+}) ion gradients across the plasma membrane. In fact, 50–60% of total brain ATP is used to maintain these gradients, especially through Na^{+}/K^{+} pumps (Erecinska and Silver, 1994).

In addition to the generation of cellular energy, mitochondria also play an important role in regulating calcium homeostasis (Babcock et al., 1997; Budd and Nicholls, 1996; Jouaville et al., 1995; Werth and Thayer, 1994) as well as in neuronal Ca^{2+} signaling. Indeed, calcium serves as a regulator of kinases, phosphatases, proteases, transcription factors and ion channels as well as an intracellular messenger for membrane excitability, exocytosis, vesicle trafficking, muscle contraction, cell proliferation, fertilization, metabolism, crosstalk between signaling pathways and apoptosis (Carafoli et al., 2001). Additionally, Ca^{2+}-sensitive dehydrogenases can regulate oxidative phosphorylation and ATP synthesis during times of high cellular demand (McCormack and Denton, 1980). Ca^{2+} uptake, sequestration and release by the endoplasmic reticulum (ER) and mitochondria – the two major Ca^{2+}-regulating organelles – play essential roles in modulating and interpreting Ca^{2+} signals.

In addition, mitochondrial calcium overload and subsequent dysfunction are thought to be critically important for triggering the cell death that follows ischemic and traumatic brain injury as well as in several neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's diseases and

amyotrophic lateral sclerosis (ALS). Given above, it is really interesting to focus the attention on mitochondrial calcium handling, and on the role that it plays in bioenergetics, organelle communication, organelle dynamics and trafficking, cell death signaling, and other equally important aspects of cell signaling.

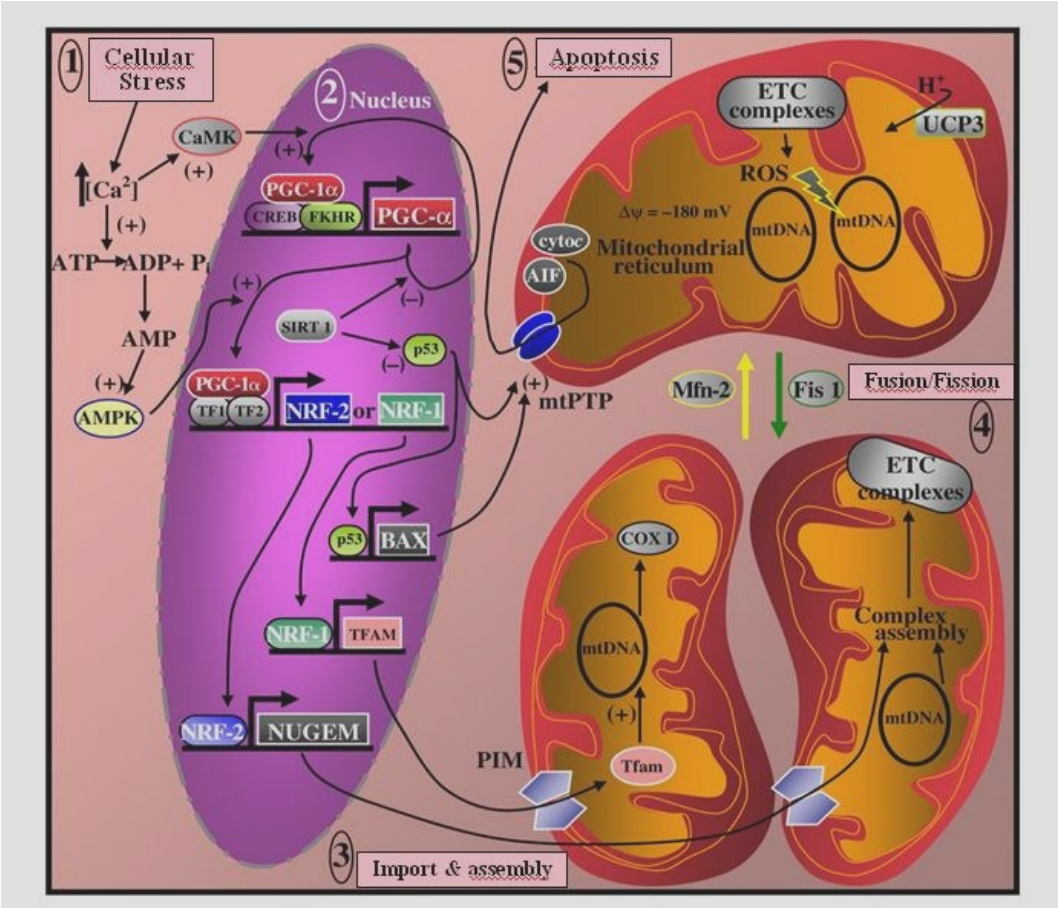

Fig. 1: Mitochondrial dynamics. Modified from Hood et al., 2006

Moreover, in a typical mammalian cell mitochondria are dynamic organelles that populate the cytoplasm and undergo continual fusion and fission event. Indeed they fuse and divide, branch and fragment, swell and extend, exist in clusters and as individual entities. Importantly, they travel throughout the cell, from the cell body outwards (anterograde movement) and in the opposite direction (retrograde movement) to deliver ATP and other metabolites where they are most required. This is seen most strikingly in highly elongated cells such as neurons: mitochondria are enriched at presynaptic terminals at the ends of axons and at postsynaptic terminals at the ends of dendrites, where bioenergetic demand is particularly high.

The inability of mitochondria to execute all these functions would be expected to disrupt cellular physiology and viability, and the degree of impairment likely corresponds to that cell's requirements for having well-functioning mitochondria positioned in the right place at the right time. For these reasons, there is growing enthusiasm for the notion that defects in mitochondrial dynamics might play a pivotal role in the pathogenesis of neurodegenerative disorders and the consequences of dynamic changes in mitochondrial morphology under physiological and injurious conditions require many further study (Frank et al., 2001; Rintoul et al., 2003; Szabadkai et al., 2004; Yu et al., 2006).

1.2 Mitochondria and calcium homeostasis

Besides performing oxidative phosphorylation, mitochondria, are able to sense and shape calcium (Ca^{2+}) transients, thus controlling cytosolic Ca^{2+}signals and Ca^{2+}-dependent proteins. Indeed, it has been well established for many years that mitochondria have a huge capacity to accumulate calcium. While the physiological significance of this pathway was hotly debated until relatively recently, it is now clear that the ability of mitochondria in calcium handling is an ubiquitous phenomenon described in every cell system in which the issue has been addressed. Therefore, mitochondria are now recognized as one of the main intracellular calcium storing organelles which play a key role the intracellular calcium signalling (Rizzuto et al., 2000). The maintenance of $\Delta\Psi_m$, strictly dependent by the holding of mitochondrial Ca^{2+} and Na^{+} within a narrow range of concentrations, is an essential requirement for calcium accumulation into mitochondria (Murgia et al. 2009). This process has enormous functional consequences both for cell physiology and for pathophysiology. Indeed, the presence of high levels of Ca^{2+} inside to the mitochondrial matrix is necessary for the right functioning of mitochondrial enzymes (Nicholls et al., 2004). Nevertheless, when calcium concentration into the mitochondria overcomes its storage capability, as happens in pathological conditions such as in neuronal

anoxia, a subsequent increase in reactive oxygen species free radical production (ROS) occurs to face the rapid rise of $[Ca^{2+}]_i$. This results in a damage of the inner mitochondrial membrane and the oxidation of the proteins involved in the electron transport, in proton pumping and in ATP production (Dugan and Choi, 1994). A further consequence of the rise of mitochondrial $[Ca^{2+}]_i$ is the increase of inner mitochondrial membrane permeability that leads to the opening of Mitochondrial Permeability Transition Pore (mPTP). This causes the release, from mitochondria to cytosol, of molecules potentially harmful for the cell such as cytocrome *c*, responsible for the impairment of cellular respiration and for induction of cell death (Atlante et al., 2003; Petrosillo et al., 2004), the apoptosis inducing factor (AIF), Smac/Diablo, and molecules with molecular weight lower than 15.000 daltons. As consequence of these events mitochondrial membrane permeability and ATP production are irreversibly affected, the mitochondrial capability to regulate intracellular calcium concentration in response to stress stimuli is impaired (Kroemer et al., 1998; Bernardi et al., 2001) and cells are committed to die.

These diverse Ca^{2+}-mediated processes, which occur over the course of microseconds to hours, are highly dependent on the spatiotemporal distribution of $[Ca^{2+}]_i$ (Berridge et al., 2000; Berridge et al., 2003). Microdomains of high $[Ca^{2+}]_i$ have been identified near Ca^{2+} channels on the plasma membrane and

endoplasmic reticulum (ER) (Brini et al., 1995). Mitochondria play an important role in regulating $[Ca^{2+}]_i$, in concert with the sarco-endoplasmic reticulum Ca^{2+}-ATPase, the plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger (Saris and Carafoli, 2005). In particular, mitochondrial Ca^{2+} uptake becomes relevant for the cells when $[Ca^{2+}]_i$ reach concentration of 400–500 nM (Nicholls and Scott, 1980).

The ionic homeostasis of mitochondria is largely maintained by mechanisms regulating the efflux or the influx of Ca^{2+} (Figure.2).

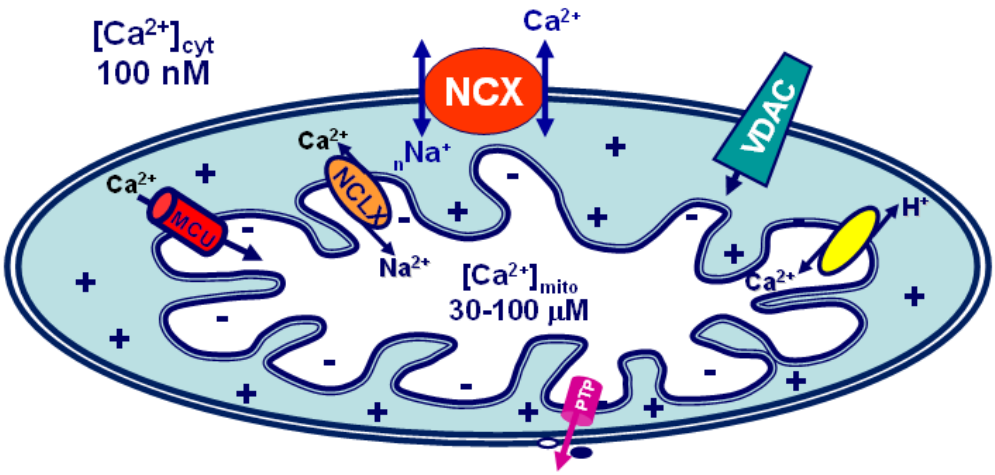

Fig. 2: Mechanisms that regulate mitochondrial calcium homeostasis.

Precisely, mitochondrial calcium uptake is primarily driven by the electrochemical potential gradient established by the mitochondrial membrane

potential and by a relatively low $[Ca^{2+}]_m$. Ca^{2+} is taken up through the mitochondrial inner membrane (IMM) by a uniporter (MCU) whose molecular identity has been recently demonstrated. In 2010, Palmer and Mootha reported that a new mitochondrial EF hand protein MICU1 (mitochondrial calcium uptake 1) was required for high capacity mitochondrial calcium uptake, and proposed that MICU acts as a calcium sensor that controls the entry of calcium across the uniporter (Perocchi et al., 2010). Building up on this discovery, two groups simultaneously identified the mitochondrial calcium uniporter in June 2011 (De Stefani et al., 2011; Baughman et al., 2011). First, using *in silico* analysis combined with phylogenetic profiling and analysis of RNA and protein co-expressed with MICU1, the group of Mootha isolated a novel protein that co-immunoprecipitated with the exogenously expressed MICU1. Then, using the same database, the group of Rizzuto independently identified the same protein. Functional analysis confirmed that this protein behaves as expected for the mitochondrial uniporter, and it was therefore assigned the defining name of MCU. This purified MCU protein is a 40 kDa protein containing two transmembrane domains localized in the inner membrane which markedly enhances mitochondrial Ca^{2+} uptake into the mitochondrial matrix, driven by an electrochemical potential gradient across the inner mitochondrial membrane,

usually estimated at -200,-180 mV, and generated either by the respiratory chain activity and by ATP hydrolysis (De Stefani et al., 2011; Gouriou et al., 2011).

The very steep voltage sensitivity of mitochondrial calcium import (Kapus et al., 1991) has interesting functional implications since, even modest mitochondrial depolarisation could profoundly affect mitochondrial calcium uptake. This may be important for the cells both in terms of the consequences for physiological calcium signalling and in terms of pathophysiology. In this way, mechanisms that cause small changes in mitochondrial potential may be surprisingly cytoprotective in pathological conditions of calcium overload (Rakhit et al., 2001).

An uptake pathway with properties distinct from those of the uniporter has also been described (Sparagna et al., 1995; Buntinas et al., 2001). This has been referred to as the rapid uptake mode (RaM). This pathway has the capacity to transfer Ca^{2+} very rapidly into the mitochondria during the rising phase of a Ca^{2+} pulse. The properties of the pathway differ in different tissues (Buntinas et al., 2001), but in heart, the pathway saturates quickly and is slow to reset after activation. However, the functional significance of the pathway remains to be established.

On the other hand, compared to the MCU and the others Ca^{2+} influx mechanism, the proteins that catalyze the efflux of this ion from mitochondria

have received much less attention. Ca^{2+} efflux is catalyzed by antiporters that drive Ca^{2+} out of the mitochondrial matrix in exchange with either Na^{+} or H^{+} (Nicholls and Crompton, 1980). Two types of exchangers have been functionally characterized in the 1970s, the Na^{+}/Ca^{2+} and the H^{+}/Ca^{2+} exchangers (Carafoli et al., 2003). These two pathway have been defined Na^{+}-independent pathway for Ca^{2+} efflux ("NICE") and Na^{+}-dependent pathway for Ca^{2+} efflux ("NCE") respectively. They have different kinetic of activation and calcium affinity (Harris et al., 1979; Lehninger et al., 1978; Ramachandran and Bygrave, 1978). The Na^{+}-independent Ca^{2+} efflux is the main mitochondrial Ca^{2+} efflux system in non-excitable cells and since no specific cations have been found to be exchanged with Ca^{2+} it is believed to be a Ca^{2+}-H^{+} exchanger (Saris et al., 2005). This transport mechanism requires transmembrane potential, since it is not observed in non-energized mitochondria, thus indicating that it is not an electroneutral passive $1Ca^{2+}$–$2H^{+}$ exchanger (Gunter et al., 1991). Indeed, this system is able to extrude Ca^{2+} against a gradient that is much higher than predicted from thermodynamics for an electroneutral H^{+}/Ca^{2+} exchanger, which indicates that it uses a component of the electrochemical gradient for its activity. A characteristic of this transporter is that it saturates at low calcium loads and its kinetics are extremely slow (Bernardi et al., 1999). This emphasizes a feature of the mitochondrial Ca^{2+} machinery: it is equipped with high V_{max} uptake transport

systems coupled to slow and easily saturable release systems, increasing the risk of Ca^{2+} overload (Murgia et al., 2009).

The molecule catalyzing mitochondrial Na^{+}/Ca^{2+} exchange has been recently identified as NCLX/ NCKX6, a protein localized in mitochondrial cristae (Palty et al., 2009), whereas stomatin-like protein 2 (SLP-2), an inner membrane protein, was shown to negatively modulate the activity of the mitochondrial Na^{+}/Ca^{2+}exchanger (Da Cruz et al., 2010). Functional evidence from knock-down and overexpression studies indicate that NCLX is an essential part of the mitochondrial sodium calcium exchanger whereas SLP-2 is an accessory protein that negatively regulates mitochondrial Ca^{2+} extrusion. The identity of these proteins remains still uncertain and this issue will be discussed in more details in the next section of this thesis work.

Another mechanism responsible of mitochondrial calcium efflux is represented by mPTP, the “permeability transition pore”, with a protein composition still under debate, although there is evidence that several outer mitochondrial membrane (OMM), mitochondrial intermembrane space (IMS) and IMM proteins are involved in its regulation. The opening of this PTP channel can be induced by intramitochondrial Ca^{2+}, while ATP, ADP, Mg^{2+} and cyclosporin A inhibit it. The identity of the PTP’s components remains elusive but several evidence indicate a role for cyclophilin D and the adenine nucleotide translocase (Schinzel

et al., 2005; Vieira et al., 2000). The physiological role of mitochondrial Ca^{2+} induced permeability is still unclear: indeed, opening of a large pore in the inner mitochondrial membrane would allow maximal Ca^{2+} flux, due to the collapse of the membrane potential and would guarantee fast Ca^{2+} release even for very small [Ca^{2+}] gradients. The PTP has also been proposed to represent a way of clearing the mitochondrial matrix of damaged or unneeded molecules; permeability transition could also provide an important pathway for inducing apoptosis or for removing damaged mitochondria (Bernardi et al. 1999).

Finally, in the last few years, it has been recognized that the OMM might also play a role in the control of mitochondrial Ca^{2+} cycling. More specifically, it serves as a significant permeability barrier not only to Ca^{2+} uptake but also to Ca^{2+}efflux. On the other hand, it should be mentioned that the voltage dependent anion channel (VDAC) located on the OMM, plays a relevant role in the regulation of Ca^{2+} permeability across this external membrane, thus favouring the activity of the specific transport systems of the inner membrane (Crompton et al., 2002). Moreover, VDAC, together with the adenine nucleotide translocase (ANT) and cyclophilin-D, might also elicit mitochondrial Ca^{2+} efflux since it is a part of the mitochondrial PTP (Bernardi, 1999, Crompton et al., 2002).

From a pathological point of view, a cellular Ca^{2+} deregulation leading to mitochondrial Ca^{2+} overload and cell death through PTP opening followed by mitochondrial swelling has been described, as final step, for many neurodegenerative diseases. For instance, Gandhi and co-workers reported that a impaired Ca^{2+} efflux from mitochondria through the mitochondrial Na^{+}/Ca^{2+} exchanger occurs in neurons lacking PINK1, a serine threonine kinase implicated in autosomal recessive early-onset parkinsonism (Gandhi et al., 2009). This led to increased Ca^{2+} uptake capacity, decreased membrane potential, and increased ROS production, all conditions leading to early triggering of the PTP opening and concomitant neuronal death (Contreras et al., 2010).

1.2.1 Mitochondrial sodium calcium exchanger

By catalyzing Na^{+}-dependent Ca^{2+} efflux, the putative mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX) plays a fundamental role in regulating mitochondrial Ca^{2+} homeostasis, oxidative phosphorylation, and Ca^{2+} crosstalk among mitochondria, cytoplasm, and the endoplasmic reticulum (ER). Although the activity of this transporter was documented more than 30 years ago, its molecular identity remained unknown. The first evidence suggesting that

mitochondria can efflux Ca^{2+} ions in exchange for Na^{+} ions was reported by Carafoli et al. in 1974. Then, the discovery of the benzothiazepine derivative CGP37157 (CGP) as a blocker of the mNCX constituted a breakthrough for the functional characterization of the mNCX (Chiesi et al., 1988). Moreover, the stoichiometry of mitochondrial Na^{+}-dependent Ca^{2+} efflux has been the subject of protracted controversy. In 2008 Kim and Matsuoka provided conclusive evidence of the direction of the mitochondrial Na^{+}/Ca^{2+} exchange: located in the inner mitochondrial membrane, the mNCX mediates the efflux of Ca^{2+} from the mitochondria coupled to the influx of Na^{+}, with a stoichiometry of $3Na^{+}/2Ca^{2+}$, suggesting that the exchanger is electrogenic Since no gene encoding for proteins belonging to the family of the Na^{+}/Ca^{2+} exchanger has been found in the mitochondrial genome (Anderson et al., 1981), the main speculation is that OMM and the IMM not possess their own endogenous proteins operating as a Ca^{2+} efflux/influx pathway and mNCX encoded by a nuclear gene, translated into the cytoplasm and then transferred to mitochondria as it occurs for the majority of mitochondrial proteins. Indeed, 90% of mitochondrial proteins are coded by nuclear genes, synthesized in the cytosol, and, subsequently, imported into mitochondria through protein translocation machineries of the outer and inner membranes (Stojanovski et al., 2003). As for most ion transporters of the inner mitochondrial membrane, identity of these proteins has been long searched.

Only recently has the NCLX exchanger, a member of the NCX family, been hypothesized to be expressed and localized in the IMM. This Li^+ sensitive protein localized within the cristae, is both phylogenetically and functionally distinct from NCX and NCKX family members. These authors also showed that NCLX participates to the mitochondrial Na^+/Ca^{2+} exchanger activity. However, the molecular mechanisms involved in NCLX expression, transport, localization, as well as its role in mitochondrial activity regulation still remain unidentified. The role of mNCX in brain ischemia is controversial but there are a lot of considerations that suggest an involvement of mNCX in this pathological event: in brain it is well known that during ischemia mitochondria release Ca^{2+} via the putative mNCX, this further depletes ATP in ischemic neurons; mNCX controls intramitochondrial Ca^{2+}, whose excessive rise is a trigger for PTP opening and apoptosis; finally, mNCX can revert its mode of operation and act as an influx pathway for Ca^{2+} ions into the mitochondrial matrix.

1.2.2 Mitochondrial calcium handling during ischemia

The ischemic failure has been ascribed to the complex interplay among multiple pathways including excitotoxicity, acidotoxicity, ionic imbalance, oxidative stress, inflammation and apoptosis, which can all lead to cell death and irreversible

tissue injury (Dirnagl et al., 1999). Brain tissue has a high metabolic rate and thus is particularly vulnerable to ischemic damage. Reduction of the cerebral blood flow reduces the delivery of oxygen and glucose to the brain tissue and, within minutes, impairs the ability of neurons to maintain ionic gradients (Martin et al., 1994). Mitochondria have been implicated as central players in the development of ischemic cell death both through impairment of their normal role in generating much of the ATP for neural cell function and as key mediators in cell death pathways. In this regards, ATP depletion, occurring in the ischemic brain, induces neuronal membrane depolarization and promotes the release of synaptic glutamate, a rise in cytosolic Ca^{2+}, the reverse operation of glutamate transporters and the consequent swelling of the cells (Choi et al., 1994; Sattler and Tymianski, 2000; Nishizawa et al., 2001; Tanaka et al., 2004). This cascade of events leads to a massive entry of calcium into the cells, which is well known to play an essential role in stroke induced cerebral damage. The increase in free cytosolic calcium is transmitted to the matrix of mitochondria by Ca^{2+} channels and exchangers located on the inner mitochondrial membrane. Moderate calcium elevations within the mitochondrial matrix increase the activity of enzymes of the tri-carboxylic cycle, therefore boosting metabolism. Excessive increases in matrix [Ca^{2+}], however, alter the permeability of mitochondria, impair their ability to generate ATP and cause the release of pro-apoptotic

factors (Sims et al., 2010). Therefore, mitochondrial dysfunctions resulting from a calcium overload have been shown to be important in the process of ischemia-induced cell death (Starkov et al., 2004). Mitochondrial changes resulting in the release of proteins are central to the intrinsic pathway. These proteins lead to the activation of caspases, particularly caspase-3 in brain, which in turn induces cellular changes including internucleosomal chromatin condensation and DNA fragmentation (Rich et al., 2000; Kroemer et al., 2007; Hengartner et al., 2000). Large mitochondrial channels promote cytochrome c release (Clem et al., 1998; Fujita et al., 1998) and synaptic failure (Jonas et al., 2003). If the mPTP, VDAC, and other proteins are involved in excitotoxity, perhaps the BCL-2 family proteins also come together in a protein complex with these mitochondrial players during ischemic injury. Indeed, patch clamp studies revealed that ischemic insults in neurons quickly produce changes in synaptic efficacy coincident with the onset of large mitochondrial ion channel activity (Jonas et al., 2003; Bonanni et al., 2006). Besides plasma membrane depolarization, the other important event in ischemia is a rise in cytosolic calcium as a result of several intracellular events (Figure 3). Indeed, the depletion of intracellular ATP slows the activity of intracellular membrane and plasma membrane calcium ATPases which are used to extrude calcium from the cell. In addition, the lack of ATP causes impairment of Na^+/K^+ exchanger activity and a consequent

depolarization of the plasma membrane. This activates voltage-gated calcium channels and induces the release of glutamate, which further acts on calcium-permeable glutamate receptors at post synaptic level to allow the influx of calcium into the cells. Moreover, the energy failure both in neurons and in glial cells leads to additional glutamate release following reversal of the glutamate uptake transporters. Finally, intracellular acidification caused by lactic acidosis also contributes to excitotoxicity by leading to increased intracellular Na^+ through Na^+/H^+ exchange, with resultant further impairment of Na^+/Ca^{2+} exchange. All these pathways contribute to the triggering of Ca^{2+} entry into neurons and, in turn, to larger than usual increases in the cytosolic calcium concentration. To avoid calcium overload, plasma membrane calcium pumps (PMCA) actively extrude calcium from the cytoplasm during neuronal activity. The increased turnover of PMCA increases the consumption of intracellular ATP that, in neurons, is mainly derived from mitochondrial oxidative phosphorylation. Cytosolic Ca^{2+} elevations are rapidly transmitted to the mitochondrial matrix, where they amplify the activity of Krebs cycle enzymes and of the ATP synthase, thereby increasing the production of ATP (Denton et al., 2009; Jouaville et al., 1999). During physiological Ca^{2+} elevations, the boost of ATP enables PMCA to extrude the cytosolic calcium and to sustain neuronal activity. Conversely, during ischemia the levels of oxygen and glucose drop rapidly

leading to impairment in ATP produced by mitochondria and by cytosolic glycolysis. As a result, ATP-dependent calcium extrusion mechanisms progressively interrupt because the continuous activity of the Na^+/K^+ ATPase depletes intracellular reservoir of ATP. The relationship between Na^+/K^+ ATPase and PMCA is directly demonstrated since the PMCA activity, collapsed during metabolic depletion, can be rescued by inhibition of the Na^+/K^+ ATPase (Castro et al., 2006). PMCA inhibition amplifies the cytosolic calcium elevations that are transmitted to the mitochondrial matrix, and can then triggers mitochondrial calcium overload, mitochondrial dysfunction, release of mitochondrial pro-apotpotic factors and activation of death signals (Starkov et al., 2004; Duchen et al., 2004; Kristian et al., 1998).

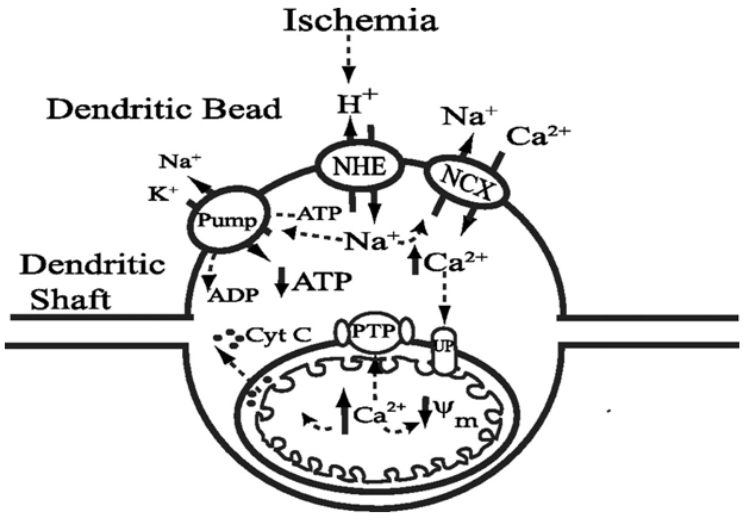

Fig. 3: Illustration of dendritic ionic disruption and mitochondrial dysregulation in ischemic neurons (Kintner et al., 2010)

On the other hand, calcium release from the endoplasmic reticulum has been associated to ischemia induced-cell damage (Paschen et al., 1999; Chen et al., 2008). Mitochondria and endoplasmic reticulum are maintained in very close proximity by linker proteins (Csordas et al., 2010; Csordas et al., 2006). Because of this proximity, the release of calcium ions through IP3 receptor of the endoplasmic reticulum readily triggers an entry of calcium in adjacent mitochondria (Giacomello et al., 2010; Rizzuto et al., 2003). Thus, neuronal mitochondria are exposed both to Ca^{2+} ions entering across membrane channels and to Ca^{2+} ions released from endoplasmic reticulum Ca^{2+} stores. Although the magnitude of this ischemia-induced mitochondrial Ca^{2+} elevation is comparable to the responses evoked by the opening of membrane channels or by the addition of Ca^{2+}-mobilizing agonists, its duration far exceeds the physiological responses. Long lasting cytosolic Ca^{2+} elevations are an essential feature of the ischemic process in neurons (Cross et al., 2010; Bano et al., 2007). This Ca^{2+} overload reflects the failure of Ca^{2+} extruding systems to cope with the excess Ca^{2+} ions that enter cells across deregulated plasma membrane channels, following the cleavage of plasma membrane Ca^{2+} pumps (Schwab et al., 2002; Pottorf et al., 2006), and Na^{+}/Ca^{2+}exchangers (Bano et al., 2005). After this global cytosolic Ca^{2+} elevation, mitochondria are exposed to micromolar Ca^{2+}concentrations for long durations in ischemic neurons, which favours Ca^{2+}

uptake by the MCU. Importantly, mitochondria start a significant calcium buffering activity whenever the cytoplasmic calcium level rises above the “set-point” for the balance of mitochondrial influx and efflux of calcium (Nicholls and Chalmers, 2004). However, acutely massive elevations in cytosolic calcium, or chronically elevated cytoplasmic calcium above the set-point, leads to calcium overload in the mitochondria and induces permeability transition, via the calcium-activated pore (mPTP) in the inner membrane. Although the causal relationship between mitochondrial Ca^{2+} accumulation and mPTP opening is well established, it is not clear exactly how or if the mPTP always gets activated during ischemia (Reynolds et al., 1999). Indeed it is clear that calcium accumulation by mitochondria impairs cellular energy: the mitochondria need to constantly keep the proton gradient during long-term calcium uptake and, therefore, in the presence of plasma membrane depolarization after glutamate exposure, the cells need more energy than that produced by mitochondria during ischemia. This condition induces mitochondrial membrane potential depolarisation, further activation of the voltage dependent mPTP, and neuronal cell death. The relationship between mPTP and neuronal death during cerebral ischemia do not directly imply the involvement of matrix Ca^{2+} accumulation (Gouriou et al., 2011). By the way, it has been hypothesized that mitochondrial Ca^{2+} overload is a consequence, rather than a cause, of the bioenergetic failure

that follows mPTP opening, suggesting that Ca^{2+} elevation represents a marker of diseased mitochondria and not the cause of the mPTP activation which occurs after reperfusion (Kim et al., 2006). Another important parameter to take into account is the timing of the mPTP opening during ischemia/reperfusion. In this regard, there is a broad consensus that in the heart, during ischemia, the factors promoting mPTP opening such as increased matrix Ca^{2+} and depolarization, are balanced by mPTP antagonists such as intracellular acidosis, high levels of Mg^{2+} and ADP, thus preventing mPTP opening (Di Lisa et al., 2011). Therefore, it is possible to speculate that the restoring of oxygen and substrate occurring during reperfusion enables mitochondria re-energized to take up the Ca^{2+} accumulated in the cytosol during ischemia and, to produce a burst in ROS. The combination of these factors provides ideal conditions for triggering mPTP opening (Halestrap et al., 2010). Whether the same sequence of events also occurs in ischemic brain is not known, and further studies are needed to determine the precise timing of the PTP opening during cerebral ischemia.

The recent identification of the proteins involved in mitochondrial Ca^{2+} uptake and release provides new opportunities to study the role of mitochondrial Ca^{2+} in neuronal death during cerebral ischemia. Targeting the proteins that control the fluxes of Ca^{2+} should reveal whether altered mitochondrial Ca^{2+} handling

is causally related to ischemic neuronal death, and can potentially increase the repertoire of therapeutic tools to treat ischemic brain diseases. In this context, a strategy to reduce mitochondrial Ca^{2+} content during ischemia might be addressed to potentiate the activity of mitochondrial efflux pathways both on the IMM and on the OMM. The molecular identification of NCLX and NCX as proteins able to regulate mitochondrial Na^{+}/Ca^{2+} exchange might result as new promising targets for the development of therapeutic strategies aimed to prevent mitochondrial dysfunction occurring during ischemia and reperfusion in the brain. However, further efforts have to be performed to improve the knowledge on the functional properties of these transporters in order to finely tune their activity to preserve mitochondrial function during stroke.

1.3 AKAP proteins

Many proteins are responsible of regulation of mitochondrial physiology. In particular, subcellular targeting through association with anchoring proteins has emerged as an important mechanism by which the cells localize signalling enzymes to sites where they can be accessed optimally by activators and, in turn, they may interact with particular substrates. Among these proteins, A Kinase Anchor Proteins (AKAPs) represent a family of non-enzymatic scaffold proteins which anchor and concentrate PKA in specific cellular and subcellular compartments (Rubin et al., 1994; Dodge and Scott, 2000; Edwards and Scott, 2000).

AKAPs are a group more than 50 proteins functionally, rather then structurally, related proteins and each contains a common RII-binding site formed by amphipathic 14-18 amino acid aligned along one face of the helix and charged residues and the other side, that bind amino termini of PKA-RII dimer (Carr et al., 1991; Newlon et al., 1997).

Although most AKAPs that have been characterized to bind to RII subunits with high affinity, several AKAPs have been reported to interact specifically with RI. RII subunits bind to AKAPs with nanomolar affinity; by contrast, RI subunits bind to AKAPs with only micromolar affinity. However D-AKAP1 and

D-AKAP2 are examples of dual-specificity AKAPs that can anchor both types of R subunit (Huang et al., 1997; Wang et al., 2001).

Each AKAP also contains a subcellular targeting domain that restricts its localization within the cell. A combination of subcellular-fractionation and immunohistochemical studies have identified AKAPs in association with a variety of cellular compartments, including centrosomes, dendrites, endoplasmic reticulum, mitochondria, nuclear membrane, plasma membrane and vesicles (Wong and Scott, 2004).

Although AKAPs have been defined on the basis of their interaction with PKA, an additional feature of many of these molecules is their ability to bind to other signalling enzymes. AKAPs form a multiprotein complex with the presence of signal transduction and signal termination enzymes in the same network. This creates focal points of enzyme activity where the bidirectional regulation of signalling events can be controlled and the phosphorylation status of target substrates is precisely regulated (Feliciello et al., 2001).

Besides kinase and phosphatase, also phosphodiesterases, the enzymes that catalyze cAMP metabolism, are present in complex with AKAP and PKA. Upon hormonal stimulation, increased cAMP levels overcome the PDE activity, releasing active PKA-C subunit from the AKAP complex. This leads to PKA phosphorylation of the tethered PDE4D3 on Ser_{54}, thereby increasing

the local PDE activity. The subsequent increase in cAMP metabolism returns cAMP levels to basal, favouring the reformation of the PKA holoenzyme (Carlisle Michel et al., 2004).

Structural data indicate that there is a single region of multiple contact sites, between the RII subunit dimer and the AKAP. AKAP-PKA complex is likely to be a constitutive interaction in cells and not subject to regulation. These findings, altogether, suggested that AKAPs serve to place the PKA holoenzyme at locations where it can respond rapidly to flow of cAMP production and to allow certain PKA phosphorylation events by placing the enzyme close to a particular subset of substrates.

1.3.1 Role of AKAP121 in the cellular hypoxia

Mitochondrial AKAP121 (also named D-AKAP1) has a major role in targeting PKA to the OMM. AKAP121, AKAP100 and AKAP84 are products of a single gene (AKAP1) that are generated by alternative RNA splicing (Lin et al., 1995; Trendelenburg et al., 1996; Chen et al., 1997; Huang et al., 1997; Huang et al., 1999; Furusawa et al., 2002). All splice variants share a similar 525 aminoacid residue NH2-terminal core, but diverge significantly at the C-terminus.

AKAP121 is widely expressed in several tissues such as germ-cell lineage, hearth and thyroid and its accumulation is regulated at the transcriptional level by the cAMP/PKA pathway (Feliciello et al., 1998).

AKAP121 is composed of different domains indicated below (Figure 4) .

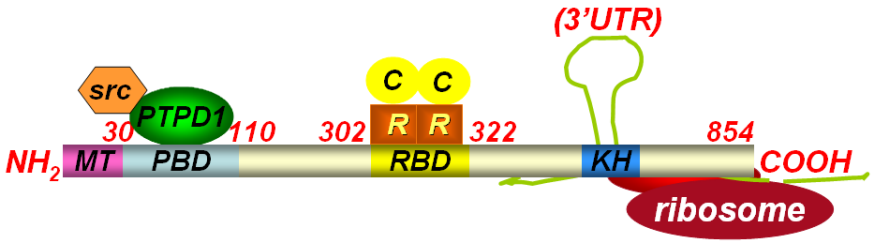

Fig. 4: AKAP121 structure

(A) The R-binding domain (RBD) of AKAP121 (residues 302-322) is identical in all splice variants and it mediates the interaction with RII at high affinity (Kd 2-8 nM) and with RI at low affinity (Kd 185 nM) (Herberg et al., 2000).

(B) The first 30 NH_2-terminal residues mediate the targeting of S-AKAP121/84-PKAII complexes to mitochondria, both in male germ-cells and in transfected heterologous cells (Lin et al., 1995; Chen et al., 1997). AKAP84 accumulates in the outer membrane of mitochondria in spermatids at a late phase of development, during the beginning of nuclear condensation and tail elongation. *De-novo* expression of AKAP84 during late spermatogenesis

coincides with the maximal expression and subsequent anchoring of RII and PKAII to mitochondria (Lin et al., 1995).

(C) Phosphatase binding domain (PBD) of AKAP121 (residues 30-110) that binds Tyrosine Phosphatase D1 (PTPD1). PTPD1 activates *src* tyrosine kinase and increases the magnitude and duration of epidermal growth factor (EGF) signalling. AKAP121 binds to and redistributes PTPD1 from the cytoplasm to mitochondria and inhibits EGF signalling. By binding and/or targeting the phosphatase on mitochondria, AKAP121 modulates the amplitude and persistence of src-dependent EGF transduction pathway (Moller et al., 1994; Cardone et al., 2004).

(D) KH domain a conserved sequence of AKAP149/AKAP121 (AKAP121 residues 565-613, AKAP149 residues 611-659) with RNA-binding capabilities (Trendelenburg et al., 1996; Chen et al., 1997).

This multicomponent system, reminiscent of other AKAP complexes at cell membranes, ensures efficient translation and import of nuclear-encoded mitochondrial proteins. It is suggested that PKA may phosphorylate some of these proteins co-translationally, as well as acting on AKAP121 itself to regulate the stability of the RNA-AKAP121 complex (Ginsberg et al., 2003; Feliciello et al., 2005). Functionally, AKAP121 by anchoring the PKA to mitochondria supports cAMP signalling and suppresses apoptosis: a cAMP

increases following adenylyl cyclase stimulation activates a membrane-anchored PKA (included Golgi-centrosome PKA, indicated as AKAP). PKA catalytic subunit dissociated from the holoenzyme enters the nucleus and phosphorylates CREB1. The rise in cAMP concentrations dissociates and activates mitochondrial-anchored PKA, which phosphorylates serine155 of the proapoptotic protein BAD. This protein is a BH3-proapoptotic Bcl-2 family member and acts at a key nodal point in the mitochondrial apoptotic pathway. Un-phosphorylated BAD binds to and inactivates antiapoptotic Bcl-2 homologs, favouring release of cytochrome C from mitochondria and inducing apoptosis. Phosphorylation by PKA blocks BAD association with Bcl-2 and inhibits apoptosis (Harada et al., 1999).

Recently it has been demonstrated that the PKA anchoring protein AKAP121 regulates the activity of the components of the mitochondrial respiratory chain, thus promoting $\Delta\Psi_m$ hyperpolarization and improving the oxidative synthesis of ATP in a PKA dependent manner (Livigni et al., 2006). Interestingly, the cell, in response to reduction in oxygen availability, degrades mitochondrial scaffold protein AKAP121. AKAP121 transmits cAMP/PKA signal to mitochondria, thus stimulating ATP synthesis. Hypoxia-induced E3–Ub ligase Siah2 was detected in complex with AKAP121. Siah2 binding leads to ubiquitination and rapid degradation of AKAP121, both *in vitro* and in intact tissue. The consequent drop

in AKAP121 concentrations significantly reduced mitochondrial activity (Carlucci et al., 2008). In higher eukaryotes, oxygen fuels mitochondrial respiration and oxidative ATP synthesis. Oxygen concentration is maintained at physiological levels by highly organized respiratory and circulatory systems. In ischemia, obstruction of blood flow to tissue leads to decrease of oxygen (hypoxia) and metabolite diffusion to cells. Hypoxia is rapidly detected by oxygen-sensing mechanisms that alter gene transcription patterns. These alterations have an important role in switching from oxidative to fermentative metabolism.

The major regulator of cellular responses to hypoxia is HIF-1α. HIF-1α is a transcription factor composed of a heterodimer of a hypoxia-inducible α-subunit and a constitutively expressed β-subunit. HIF-1α induces expression of a number of genes, including that of vascular endothelial growth factor, transforming growth factor-β and erythropoietin, which are involved in vascularization, erythropoiesis, metabolism and other central cellular processes. Under normoxic conditions, HIF-1α hydroxylation by PHD2 promotes HIF-1α binding to the von Hippel–Lindau complex and rapid degradation by the Ub–proteasome pathway (Berra et al, 2003). Hypoxia induces expression of Siah2, which carries an N-terminal RING domain followed by two zinc-finger motifs and a C-terminal substrate-binding domain. The RING finger domain of Siah2 mediates transfer of Ub monomer from E2 Ub–ligase to PHD1/3, promoting its

proteasomal degradation. As a result, HIF-1α accumulates and activates transcription of hypoxia-induced genes (Hu et al, 1997; Nakayama et al, 2004). Recent findings demonstrated a new mechanism that the cells utilize to adapt physiologically to oxygen deprivation. This involves regulation of components of the signal transduction pathway that controls oxidative respiration at the post-translational level. Indeed Siah2 induces ubiquitination and proteasomal degradation of the scaffold protein AKAP121, thus lowering the basal activity of mitochondrial respiration in hypoxic cells. The cells exposed to hypoxia or overexpressing Siah2 show an AKAP121 degradation accompanied by a significant decrease in $\Delta\Psi_m$ and mitochondrial metabolic activity. In view of the ubiquitous expression of AKAP121, this regulatory system might likely be used as a rapid and efficient way to attenuate oxidative metabolism during hypoxia in most, if not all, tissues. In particular, AKAP121 is also expressed in discrete brain areas, including hippocampus and cortex, as well as in the corpus striatum and cerebellum. Interestingly, middle cerebral artery occlusion consistently decreased AKAP121 levels specifically in the ischaemic cortical area.

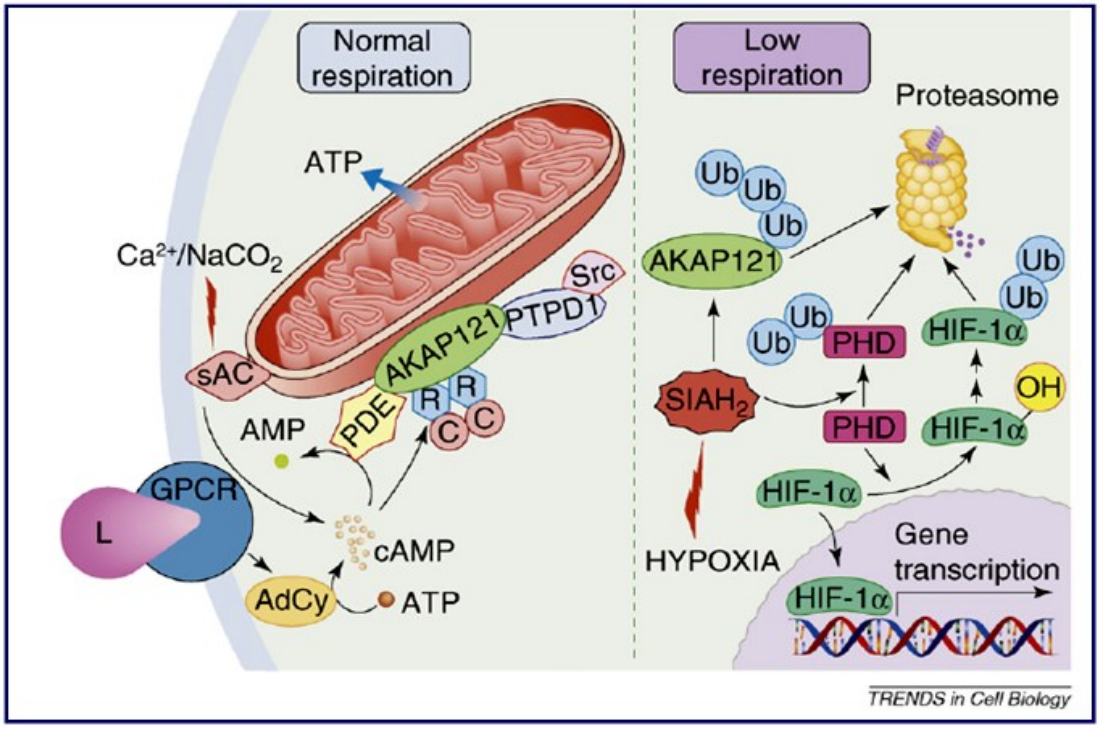

Fig.5: Role of AKAP 121 in hypoxia (Carlucci et al., 2008)

1.4 Mitochondrial fusion and fission

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. 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. 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

Many studies suggest that changes in the mitochondrial shape can affect a variety of biological processes. Mitochondria change their morphology by undergoing fusion or fission and the fine balance between these two opposing reactions 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).

In a typical mammalian cell, the mitochondria are highly dynamic and undergo continual fusion and fission (Chen et al., 2005). These processes control not only

the overall morphology of the mitochondrial population, but also its proper function. Three proteins have been shown to be central to the fusion of mammalian mitochondria. The mitofusins, Mfn1 and Mfn2, are essential GTPases localized to the mitochondrial outer membrane (Chen et al., 2003; Santel et al., 2001). Deletion of either Mfn1 or Mfn2 results in mitochondrial fragmentation, although low levels of mitochondrial fusion remain. Deletion of both mitofusins abolishes all mitochondrial fusion (Koshiba et al., 2004; Chen et al., 2005). Mitofusins are required on adjacent mitochondria during fusion and form complexes in trans that tether mitochondria together. The third protein required for fusion is OPA1, a dynamin-related GTPase (Cipolat et al., 2004; Chen et al., 2005). OPA1 is localized to the intermembrane space, with tight association with the inner membrane (Griparic et al., 2004; Satoh et al., 2003).

Separate machinery mediates mitochondrial fission. Fission requires Fis1, a mitochondrial outer membrane protein, and Drp1, another dynamin-related GTPase (Chen et al., 2005). Inhibition of either protein results in elongation and increased interconnectivity of mitochondrial tubules (Lee et al., 2004; Smirnova et al., 2005). Whereas Fis1 is localized uniformly on mitochondria, Drp1 oligomerizes into puncta on the mitochondrial surface, and some of these puncta proceed to mitochondrial fission. Both fusion and fission are important for mitochondrial function. When mitochondrial fusion is completely abrogated, by

removal of mitofusins or OPA1, cells grow poorly and have greatly reduced respiratory function (Chen et al., 2005). Clearly, these results indicate that mitochondrial fusion is important for the health of the mitochondrial population and of the entire cell. Mitochondrial dynamics is thought to protect mitochondrial function by allowing the mixing and exchange of small molecules, proteins and mtDNA (Chen et al., 2004). Mutations in mitochondrial fusion proteins would lead to mitochondrial dysfunction by preventing this ability of mitochondria to cooperate. In addition, mitochondrial morphology defects have secondary effects on the transport of mitochondria. Finally, fission has been shown to be an important component of the mitochondrial apoptosis pathway (Youle et al., 2005). In many models of programmed cell death, mitochondria fragment during the early stages of apoptosis, and inhibition of Drp1 or Fis1 can ameliorate cell death. In contrast, mitochondrial fusion molecules can demonstrate protective effects in programmed cell death (Youle et al., 2005; Sugioka et al., 2004). Given the above, it is not surprising that defects in mitochondrial dynamics lead to neurological disease. Remarkably, mitochondrial dysfunction is considered to be one of the key event linking ischemic/recirculation insult with neuronal cell death (Berridge et al. 2003). Moreover, recent studies showed that in a cardiac cell model of ischemia, mitochondria undergo fragmentation, a process that is dependent on the mitochondrial fission protein dynamin-related protein 1 (Drp1)

(Ong et al., 2011), suggesting a role of Drp1 also in the pathogenesis of cerebral ischemia/reperfusion.

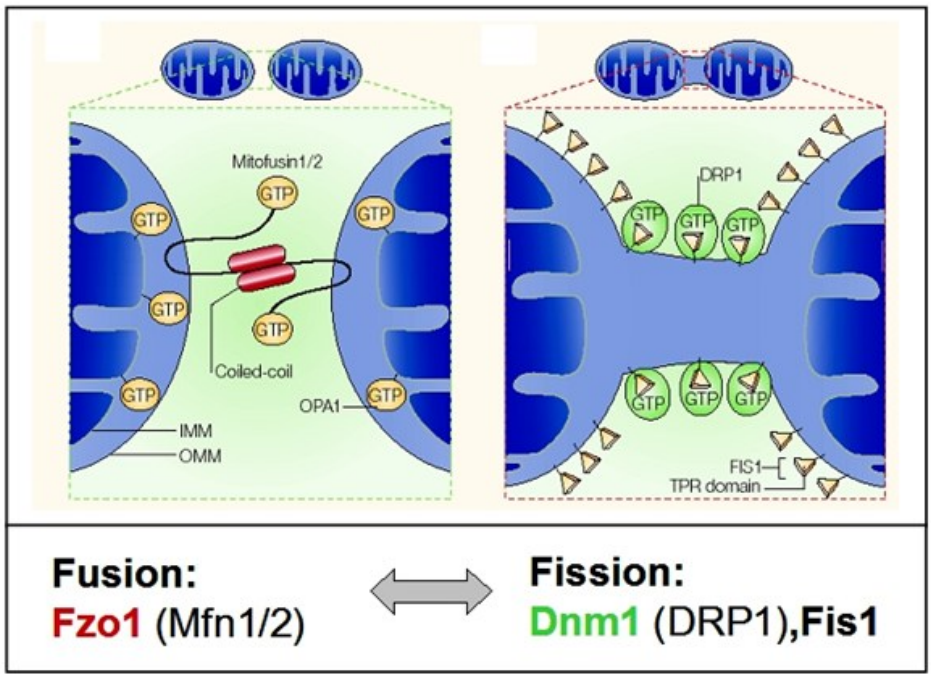

Fig.6. Mitochondrial fusion and fission proteins on the OMM (Youle and Karbowsky, Nat Rev Mol Cell Biol. 2005)

The balance between fusion and fission requires tight control of the factors that participate in mitochondrial dynamics. The activity of these proteins can be modulated by covalent modifications, such as phosphorylation, sumoylation and ubiquitylation (Carlucci et al., 2008). The mitochondrial fusion and fission machinery is tightly controlled by protein phosphorylation. In particular, post-translational regulation of Drp1 is an active area of research, with ubiquitylation, sumoylation, and phosphorylation having been documented (Chang and

Blackstone, 2007; Cribbs and Strack, 2007; Han et al., 2008; Harder et al., 2004; Nakamura et al., 2006; Wasiak et al., 2007). Indeed, Drp1 is a direct substrate of PKA: β-adrenergic stimulation of heart muscle by isoproterenol in vivo or PKA activation increases phosphorylation of hDrp1 within the GTPase effector domain (GED) at Ser637 and inhibits its GTPase activity (Cribbs et al., 2007). This down regulation of activity probably reflects the inability of the phosphorylated GED domain to interact intramolecularly with the Drp1 GTP-binding motif. The resulting inactivation of Drp1 promotes elongation of mitochondria and enhances cellular resistance to pro-apoptotic stimuli. On the other hand, membrane depolarization or L-type calcium channel agonists promote de-phosphorylation of Drp1 phospho-Ser637 by the calcium-activated Ser/Thr phosphatase calcineurin (also known as PP2B) (Chang et al., 2007; Cribbs et al., 2007). The subsequent calcium-dependent de-phosphorylation restores Drp1 GTPase activity and induces 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. This type of regulation, based on protein modification, can rapidly adapt mitochondrial activity to changes in metabolic demands and nutrients availability.

2.AIMS OF THE STUDY

In the first step of the present study we examined (a) whether the nuclear encoded NCX family proteins are present on mitochondria and, if they are, (b) where they are localized, (c) what might be their contribution to mitochondrial Ca^{2+} handling and, finally, (d) whether NCX activity on mitochondria is controlled by AKAP121, a member of the AKAP family proteins in the outer mitochondrial membrane (Carlucci et al 2008; Livigni et al 2006).

In order to identify whether one or all three isoforms were present at mitochondrial level, molecular biology experiments were performed on mitochondrial extracts obtained from BHK cells transfected with each isoform of the sodium/calcium exchanger (NCX), NCX1, NCX2 and NCX3. These experiments identified the presence of only isoform 3 of the sodium/calcium exchanger (NCX3) on the mitochondrial membrane. These observations were supported by immunocytochemistry experiments using specific fluorescent antibodies against NCX and specific mitochondrial markers. In addition, using a biochemical approach, it was demonstrated that NCX3 is present on the outer mitochondrial membrane. Further studies were designed to clarify the functional role of the Na^{+}/Ca^{2+} exchanger in the regulation of mitochondrial ionic homeostasis and the possible involvement of AKAP121 in the modulation of this

function. This working hypothesis has been supported by experimental evidence that proteins belonging to the NCX family show multiple phosphorylation sites, both for the PKA and for PKC, which would, therefore, seem be involved in regulating the activity of this exchanger, both in response to physiological and pathological stimuli (Blaustein and Lederer, 1999; Schulze et al., 2003). Since the AKAP121 protein is responsible to target the PKA to mitochondria, it turns out to be a potential regulator of mitochondrial calcium concentrations by modulating the NCX activity. To verify this hypothesis, we measured mitochondrial calcium levels using confocal microscopy techniques in BHK cells stably transfected with NCX3 which endogenously express AKAP121 and BHK wild-type cells (WT) transiently co-transfected with NCX3 and AKAP121. In these cells, immunocytochemistry experiments and immunoprecipitation techniques showed that NCX3 is present in mitochondria and co-localize with AKAP121 through direct physical interaction. Functional experiments were carried out by measuring the mitochondrial calcium concentration ($[Ca^{2+}]_{mito}$) in BHK cells transiently transfected with NCX3 and co-transfected with AKAP-121. Under these conditions the obtained results showed that the basal $[Ca^{2+}]_{mito}$ was lower than that measured in BHK WT cells or BHK cells transiently transfected with NCX3. This effect was abolished when cells were transiently transfected with NCX3 and $AKAP121_{L313\text{-}319P}$ mutant, unable to bind PKA, or with PKI

construct, able to specifically inhibit PKA. On the other hand, evidence that mNCX3 is involved in Ca^{2+} extrusion is that the Ca^{2+} lowering effect, found in cells transfected with NCX3, was completely prevented by the benzothiazepine compound CGP-37157, a selective mNCX inhibitor (Cox et al., 1993; Nicolau et al., 2009). Consistently, when BHK cells either transfected with $NCX3^{F}$ or co-transfected with $NCX3^{F}$+AKAP121 were treated with CGP-37157, $[Ca^{2+}]_m$ were higher than those observed in untreated cells. The obtained results supported the hypothesis that AKAP121 was able to control $[Ca^{2+}]_{mito}$ by regulating the activity of NCX3 on the outer mitochondrial membrane. It has also been explored the hypothesis that AKAP121 may play a role in the regulation of the mitochondrial NCX3 under cellular stress conditions such as hypoxia or treatment with calcium ionophores, such as ionomycin. In both experimental conditions mitochondrial calcium levels measured in BHK WT cells were higher than those measured in BHK cells transiently transfected with NCX3 or co-transfected with NCX3 + AKAP121. These data suggest that in cellular stress conditions NCX3 could work extruding calcium from the mitochondria and that this effect could be modulated by PKA through AKAP-121. Indeed, the cells transiently co-transfected with siRNA directed against NCX3 and AKAP-121 or with the mutant $AKAP121_{L313\text{-}319P}$ showed $[Ca^{2+}]_{mito}$ similar to that observed in BHK WT cells. Moreover the results obtained in neurons exposed to Oxygen

and Glucose Deprivation (OGD) and OGD/Reoxygenation (Rx) demonstrated that also the endogenous mNCX3 plays a relevant role in the regulation of mitochondrial Ca^{2+}extrusion. Indeed, $[Ca^{2+}]_m$ significantly increased when neurons were exposed to OGD, a condition in which NCX3 expression was reduced. On the other hand, $[Ca^{2+}]_m$ decreased following OGD/Rx, a condition in which NCX3 expression returned to the basal levels. Interestingly, when NCX3 was knocked down, an impairment in mitochondrial Ca^{2+} extrusion was recorded both under basal and OGD/Rx conditions, whereas no alteration in mitochondrial Ca^{2+}extrusion occurred during OGD. These results might be related to changes in the expression of endogenous NCX3 and AKAP121 during OGD and OGD/Rx (Sirabella et al., 2009; Carlucci et al., 2008). Such finding led to the hypothesis that the interplay between mNCX3 and AKAP121 contributes to cell survival and the interaction between these two proteins appears to have a crucial role in preventing hypoxic cell death.

A second step of the study was addressed to investigate the molecular mechanisms that regulate mitochondrial biogenesis in cellular models of cerebral ischemia with particular regard to mitochondrial fusion and fission events in cells exposed to hypoxic insult. To this aim, experiments were performed in cellular models of *in vitro* ischemia using primary cultures of hippocampal and cortical neurons exposed to a combined protocols of OGD

(Scorziello et al., 2005) or chemical hypoxia (Secondo et al., 2007) followed by a period of reoxygenation. Changes in mitochondrial morphology, mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial calcium concentration were measured by using specific probes and confocal microscopy. The obtained data showed that alteration in mitochondrial morphology, an increase in $[Ca^{2+}]_{mito}$ and an impairment in $\Delta\Psi_m$ occurred in cells exposed to hypoxia. To evaluate the involvement of the mitochondrial fusion and fission proteins in the pathogenesis of ischemic neuronal death we performed Western Blotting experiments on lysates obtained from hippocampal and cortical neurons exposed to 3 and 6 hours of OGD. The obtained results showed that during OGD an increase of the expression levels of Dynamin Related Protein 1 (Drp1), a protein involved in the mitochondrial fission phenomenon, and a reduction of the expression levels of Mitofusin 2 (Mfn2), crucial protein of the mitochondrial fusion event occurred. Further objective of this project was to verify whether the protein AKAP121 could participate in the molecular events underlying the process of mitochondrial fusion and fission during ischemia. Since it was recently shown that AKAP121 is rapidly degraded in cells exposed to hypoxia by a mechanism mediated by the activation of SIAH2, we used neurons obtained from transgenic mice Siah2 -/- to evaluate alterations in the mechanisms that regulate the metabolic mitochondrial activity. Finally, due to the evidence that mitochondrial calcium homeostasis

seems to be involved in the alteration of mitochondrial dynamics during the neuronal ischemic death, the next step of the study was to verify the role of NCX3 and the putative interaction between this protein and AKAP121 in mitochondrial fission and fusion during ischemia. To this aim the neurons were exposed to OGD, a condition in which NCX3 and AKAP121 expression were reduced, followed by OGD/Rx, a condition in which NCX3 and AKAP121 expression returned to the basal levels (Carlucci et al., 2008; Sirabella et al., 2009). Interestingly, after the reoxygenation the reduction of mitochondrial FormFactor and AspectRatio values observed during OGD returned to the basal values. These results might be related to changes in the expression of endogenous NCX3 and AKAP121 during OGD and OGD/Rx, suggesting the involvement of these two proteins in the alteration of mitochondrial morphology observed during hypoxia.

Understanding the mechanisms that regulate the mitochondrial metabolic activity during the cellular response to hypoxia, the characterization of the molecular mediators involved in mitochondrial fusion and fission events and the identification of ligands that can modulate these phenomena might be useful to set new strategies for the treatment of cerebral ischemia.

3. EXPERIMENTAL PROCEDURES

3.1 Cell Culture

Wild type and stably transfected BHK cells with canine cardiac NCX1, rat brain NCX2, or NCX3 (Linck et al., 1998) were grown on plastic dishes in a mix of DMEM and Ham's F12 media (1:1) (Gibco, Invitrogen, MI, Italy) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma, St. Louis, Missouri, USA). Cells were cultured in a humidified 5% CO_2 atmosphere; the culture medium was changed every two days. For confocal and calcium imaging experiments, cells were plated on glass coverslips (Fisher, Springfield, NJ, USA) coated with poly-D-lysine (100 µg/ml) (Sigma, St. Louis, Missouri, USA), and used at least 24- hrs after seeding.

Mixed cultures of cortical neurons from Wistar rat pups, 2–4 days old, were prepared by modifying a previously described method (Abramov et al., 2007). The tissue was minced and trypsinized (0.1% for 15 min at 37°C), triturated, and plated on poly-D-lysine-coated coverslips and cultured in Neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen) and 2mM L-glutamine. For immunocytochemistry experiments, cells were plated at concentration of $1.8x10^6$ on 25-mm glass coverslips. Cultures were maintained at 37°C in a humidified atmosphere of 5%CO2 and 95% air, fed twice a week, and maintained for a minimum of 10 days before experimental use.

Primary cultures from NCX3 -/- mice were obtained as previously reported (Sokolow et al., 2004; Molinaro et al., 2008).

3.2 Plasmids and transfection

Mouse pCEP4-AKAP121 cDNA was a gift from Dr C. Rubin (Albert Einstein College of Medicine, NY). Vectors encoding CMV promoter, AKAP-121 protein, and AKAP-121 mutant lacking PKA binding activity ($AKAP121_{L313\text{-}319P}$) have been previously described (Affaitati et al., 2003). PKA activity was fully inhibited by co-transfecting a specific PKA inhibitor (PKI) which contains a PKA pseudophosphorylation site as previously described (Orellana et al., 1993). OmicLink Expression M14 vector expressing NCX3-Flag was engineered by ligating an oligonucleotide encoding a 3XFLAG epitope to the carboxy terminus of mouse NCX3 cDNA (GeneCopeia). No deletion mutant of-length NCX3 was used. In order to knocking down NCX3, the nucleotide sequence corresponding to the first nucleotide of the start codon (+124–142) of rat NCX3 (GenBank accession no.U53420) was inserted in the mammalian expression vector pSUPER.retro.puro (OligoEngine). All these constructs were transiently transfected using Lipofectamine 2000 (Invitrogen) together with the plasmid vector for the green fluorescent protein (GFP) (0.5 μg). The siRNAs were transiently transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 250 pmol/ml of culture medium.

3.3 Mitochondrial Extracts

- Extraction via differential centrifugation method

BHK-Wt and NCX3 transfected cells were lysed in a buffer solution (Buffer A) containing the following (mM): 250mannitol, 0.5EGTA, 5HEPES (pH7.4), 1.5MgCl2, 0.1%aprotinin, 0.7mg/ml pepstatin, and 1µg/ml leupeptin. Lysates were passed through a 26-gauge needle ten times. Samples were centrifuged twice, (2000rpm, 5min; 13000rpm, 10min). After the first centrifugation pellets, corresponding to the fraction that contains membranes but does not contain the intracellular organelles including mitochondria, was separated from supernatants and dosed for proteins. Supernatants, corresponding to the cytosolic fraction containing the organelles, were further centrifuged (2000rpm, 5min) to separate mitochondria from cytosolic fraction. Supernatants (Cytosol) were removed and dosed for proteins. The pellets containing mitochondria were then lysed in 50µl of lysis buffer containing (mM) 20Tris-HCl (pH7.5) , 10NaF, 150NaCl, 1PMSF, 1% NONIDETP40, 1Na3VO4, 0.1%aprotinin, 0.7mg/ml pepstatin, and 1µl/mg leupeptin, and kept on ice for 15min. Finally, samples were purified again by centrifugation (13000rpm, 10min) and supernatants (mitochondria) were dosed for proteins by Bradford's (Bradford et al., 1976) assay. The three fractions obtained were used for Western Blot(WB). The purity of mitochondrial preparation was assessed by evaluating the expression of the proteins: GM131

a Golgi marker, calnexin, an endoplasmic reticulum marker, and LAMP1, a lysosomes marker on the fractions relative to membranes, cytosol, and mitochondria

- Extraction via Percoll gradient method

The Percoll gradient method was used to purify mitochondria from tissues. C57 mice and ncx3 -/-, previously anesthetized with halothane, were killed by decapitation, and the brains were rapidly removed. Each brain was incubated twice in cold phosphate buffer solution (PBS) and then homogenized in 5ml of buffer A. The homogenate was passed through a 22-gauge needle once and then through a 26-gauge needle five times. Subsequently, it was centrifuged four times for 5 min at 2000 rpm and once for 10 min at 13000 rpm. The supernatant was removed and dosed for proteins. The pellet, containing mitochondria, was suspended in 1ml buffer A and then stratified in a 3ml solution containing: 30% v/v Percoll, 250mM mannitol, 0.5mM EGTA, 5mM HEPES (pH 7.4), and centrifuged in a Sorvall centrifuge at 9,500g for 30 min at 4°C. The fraction containing mitochondria, lying under the brown Percoll band, was removed, suspended in buffer A, and, then, centrifuged twice at 8000 rpm for 10 min. The pellet obtained was re-suspended in a 50µl NCX lysis buffer, kept on ice for 10 min and then centrifuged at 13000 rpm. The supernatant was dosed for proteins by Bradford's assay and then used for Western Blot analysis.

3.4 Immunocytochemistry

BHK-WT, and BHK cells stably transfected with NCX3 isoform and cortical neurons were cultured on glass coverslips (BHK cells for 48 h and cortical neurons for 11 DIV). The cells were rinsed twice in cold 0.01 M saline phosphate buffer at pH 7.4 (PBS) and fixed at room temperature in 4% (w/v) paraformaldheyde (Sigma, Milan, Italy) for 20 minutes. Following three washes in PBS, cells were blocked in PBS containing 10% FBS and the following antibodies: anti-NCX3 rabbit (kindly supplied by Dr. Philipson, dilution 1:4000), anti-MnSOD mouse (Upstate, dilution 1:200), anti-Flag mouse (Sigma, dilution 1:500), and anti-AKAP121 rabbit (Carlucci et al., 2008); dilution 1:100). The cells were then incubated overnight at 4°C. Next, slides were washed in PBS, incubated with anti-rabbit cy2 antibody (Jackson; dilution 1:200) and anti-mouse cy3 antibody (Jackson; dilution 1:200) for 1 hr at room temperature (25°C) under dark conditions, and washed again with PBS. Finally they were mounted onto Slow fade^{TM} antifade (Invitrogen-Molecular Probes) and analysed by confocal microscopy.

In some experiments, cells were incubated with Mitotracker Red CM XROS (20nM, 37°C for 30 min) before fixation to stain mitochondria in live cells.

Cells were analyzed for co-localization between Mito (red) and NCX3 (green) by using the “co-localization highlighter” plug-in for ImageJ Software (NIH,

Bethesda, MA, USA). Before co-localization analysis threshold settings for each image were determined and quantification was achieved by counting the number of NCX3/Mito co-localized points (white) per microscope field. Results were expressed as a percentage of colocalization.

3.5 Western Blot

***- NCXs detection*.**

Protein samples (50 μg) were analyzed on 8% 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% not 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 1:1000 antibody for NCX1 (polyclonal rabbit antibody, Swant), 1:1000 antibody for NCX2 (polyclonal rabbit antibody, Alpha Diagnostic), and 1:5000 antibody for NCX3 (polyclonal rabbit antibody, Philipson's Laboratory). The membranes were washed with 0.1% Tween 20 and incubated with the secondary antibodies for 1 h (1:5000; Amersham). Immunoreactive bands were detected with the ECL (Amersham). Discrimination among the distinct types of extracts was ensured by running parallel Western Blots with the endogen protein tubulin (localized to cell membrane), Mn-SOD (localized into mitochondrial matrix), VDAC (outer

mitochondrial membrane) or COX4 (inner mitochondrial membrane). The optical density of the bands was determined by Chemi Doc Imaging System (Biorad).

- Immunoprecipitation and immunoblot analysis.

Cells were homogenized in lysis buffer containing (50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 % Triton X-100, 100 mM NaF, 100 mM Na_3VO_4, 5 µg/ml aprotinin, 10 µg/ml leupeptin and 2 µg/ml pepstatin). The lysates were cleared by centrifugation at 15,000 x g for 15 min. Cell lysates (2 mg) were immunoprecipitated with anti-Flag mouse antibody (1:100). An aliquot of cell lysate (100 µg) or immunoprecipitates were resolved by SDS-PAGE gel and transferred onto nitrocellulose membrane. Immunoblot analysis was performed using anti AKAP-121 antibody, as previously described (Cardone et al., 2004). Chemio-luminescent (ECL) signals were quantified by Chemi Doc Imaging System (Biorad).

3.6 Immuno-Electron Microscopy analysis

Cells were fixed with a mixture of 4% paraformaldehide and 0.05% glutaraldehyde, labeled with a polyclonal antibody against NCX3 using the gold-enhance protocol, embedded in Epon-812, and cut in thin sections (Polishchuk et al., 2003). EM images were acquired from thin sections using a FEI Tecnai-12 electron microscope equipped with an ULTRA VIEW CCD digital camera (FEI, Eindhoven, The Netherlands). Surface gold density (arbitrary units, au) was

estimated according to Griffiths and Hoppeler method (Griffiths et al., 1986). According to this method a morphometric grid with definitive size (100 nm) was applied to all images acquired and then the number of gold particles present on membranes of interest (mitochondria, ER, PM) was counted. Next, the number of intersections between the organelle membranes and the grid was calculated. In the case of mitochondria only outer membrane was calculated. Finally, the number of gold particles was divided by the number of intersections to derive the gold density value. Thirty structures were analyzed for each compartment of interest. Surface gold density for NCX3 in mitochondria corresponded to 0.19 AU in neurons and 0.21 AU in BHK- transfected cells; surface gold density for NCX3 in ER corresponded to 0.24 AU in neurons and to 0.32 AU in BHK-transfected cells and finally, in plasma membrane surface gold density for NCX3 corresponds to 0.23 AU in neurons and 0.98 AU in BHK-transfected cells.

3.7 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 510 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.

3.8 Imaging cytosolic Ca^{2+}

$[Ca^{2+}]_i$ was measured by single cell computer-assisted video imaging (Secondo et al., 2007). In brief, cells, grown on glass coverslips, were loaded with 5 μM Fura-2 acetoxymethyl ester (Fura-2AM) for 30 min at room temperature in normal Krebs solution containing (in mM): 5.5 KCl, 160 NaCl, 1.2 MgCl2, 1.5 CaCl2, 10 glucose, and 10 Hepes–NaOH, pH 7.4. At the end of the Fura-2AM loading period, the coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted onto a Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a FLUAR 40X oil objective

lens. The experiments were performed as previously reported (Secondo et al., 2007).

3.9 Chemical hypoxia (CH/HYP)

Chemical hypoxia was reproduced by treating cells with 5μg/ml oligomycin and 2mM 2-DG in a medium without glucose and containing (in mM): 145 NaCl, 5.5 KCl, 1.2 MgCl2, 1.5 $CaCl_2$, and 10 Hepes, pH 7.4, for 45 min as already described (Secondo et al., 2007). Control cells were exposed to normal Krebs solution composed of (in mM): 145 NaCl, 5.5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 Glucose, and 10 Hepes, pH 7.4 for 45 min.

3.10 Combined oxygen and glucose deprivation and reoxygenation (OGD)

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% N2 and 5% CO2 and containing NaCl 116 mM, KCl 5.4 mM, MgSO4 0.8 mM, NaHCO3 26.2 mM, NaH2PO4 1 mM, CaCl2 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% N2 and 5% CO2). These experimental conditions induced 30% decrease of pO2 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% O2 and 5% CO2 for 10 min. Reoxygenation was achieved by returning neurons to normoxic conditions (37°C in a humidified 5% CO2 atmosphere) for 24 hrs.

3.11 Determination of cell death: Propidium Iodide assay

Cell death was evaluated by measuring the ratio between dead and living cells. To quantify cell death after the experimental procedures, the cells were washed with normal Krebs and double stained with 36μM Fluorescein Diacetate (FDA) and 7μM Propidium Iodide (PI) for 5 min at 37°C in a phosphate buffer solution. Stained cells were examined immediately with a standard inverse fluorescence microscope at 480 nm and 546 nm (Secondo et al., 2007). PI- and FDA-positive cells were counted in three representative high power fields of independent cultures and cell death was determined by the ratio of the number of PI positive cells/PI + FDA-stained positive cells (Wei et al., 2000).

3.12 Quantification of ATP content

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. After centrifugation at 10,000×g for 60 s, samples were diluted at 1:50 in dilution buffer (Sigma, FL-AA). To obtain bioluminescence measurements with a standard luminometer,

100μl of supernatant was mixed with 100μl of luciferin–luciferase solution. The standard curve of ATP was obtained by serial dilution of 2μM ATP solution (Maeda et al., 2003).

3.13 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 analysis of variance followed by Newman-Keuls test. Statistical significance was accepted at the 95% confidence level (P ±0.05).

4.RESULTS

4.1 Biochemical identification and localization of NCX3 isoform on the OMM

Western blot experiments on mitochondrial fractions obtained from BHK cells stably transfected with each of the three isoforms of NCX were firstly performed in order to identify which NCX isoform might be present on mitochondria. The results obtained demonstrated that NCX3 protein, apart its presence at the plasmamembrane (PM) level, was also localized on mitochondria membranes (Figure 7A), whereas NCX1 and NCX2 isoforms were not detected (Figure 7C-D). In particular, experiments performed on whole mitochondria or mitochondrial fraction of BHK-NCX3 transfected cells demonstrated that NCX3 is located on the outer mitochondrial membrane. Indeed, when the outer mitochondrial membrane was removed by treatment with the detergent TritonX-100 (0.2%) or with Trypsin (20 μg/ml), or with TritonX-100+Trypsin (Sardanelli et al., 2006), NCX3 immunoreactivity disappeared. Instead, Mn-SOD immunoreactivity, a marker of mitochondrial matrix, and COX-4 a marker of IMM, remained unaffected (Figure 7B).

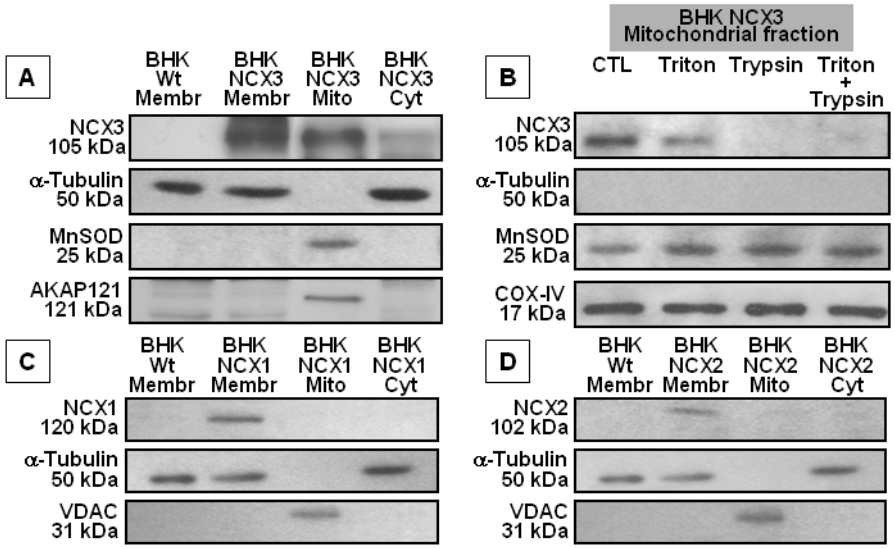

Fig. 7. **Biochemical identification and localization of NCX3 isoform on the outer mitochondrial membrane in BHK transfected cells. A**, Expression of NCX3 within membrane, cytosolic, and mitochondrial fractions obtained from BHK-WT and stably transfected BHK-NCX3 cells**. B,** Localization of NCX3 isoform on the outer mitochondrial membrane. Whole mitochondria of stably transfected BHK-NCX3 cells were digested with trypsin (2μg/100μl) in the absence or in the presence of Triton X-100 (0.2% v/v) as indicated. **C-D**, Expression of NCX1 (C) and NCX2 (D) within membrane, cytosolic, and mitochondrial fractions obtained from BHK-WT and stably transfected BHK-NCX1 or BHK-NCX2 cells, respectively.

This finding was supported by electron microscopy experiments performed in NCX3 BHK transfected cells (Figure 8) demonstrating that NCX3 is present for 98% in the plasmamembrane, for 21% on the OMM and for 32% in ER.

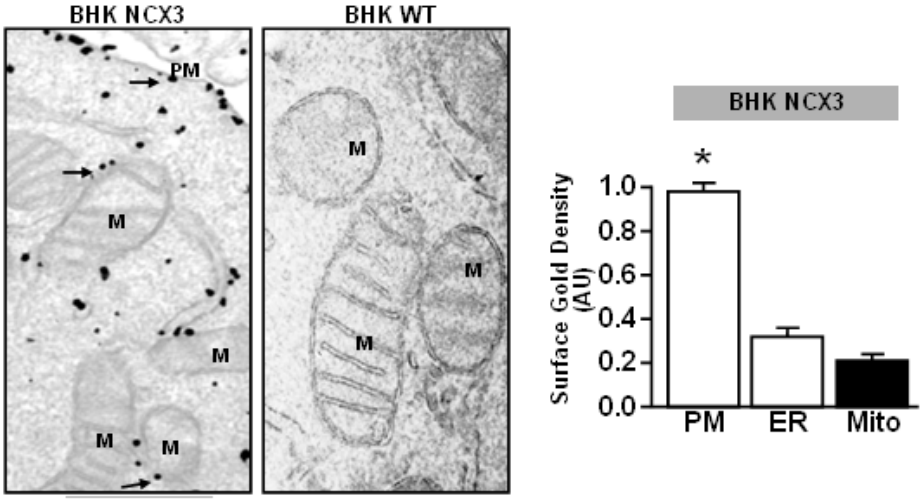

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Fig.8: Distribution of NCX3 in BHK-Wt and in BHK-NCX3 stably transfected cells along the PM, ER and mitochondria (arrows). In mitochondria gold particles can be detected exclusively on OMM. Surface gold density for NCX3 in mitochondria in BHK-transfected cells corresponded to 0.21 AU, in ER to 0.32 AU and, finally, in plasmamembrane to 0.98 AU.

In order to exclude the presence of ER contamination and to validate the purity of mitochondrial preparation, Western Blot analysis on mitochondrial extracts from NCX3 BHK transfected cells were performed using antibodies against different markers specific for ER (calnexin), for Golgi apparatus (GM131), for lysosomes (LAMP1) and for mitochondria (DRP1 and COXIV). The results of these experiments confirmed the absence of calnexin positivity on mitochondrial fraction, excluding therefore, the presence of ER contamination (Figure 9).

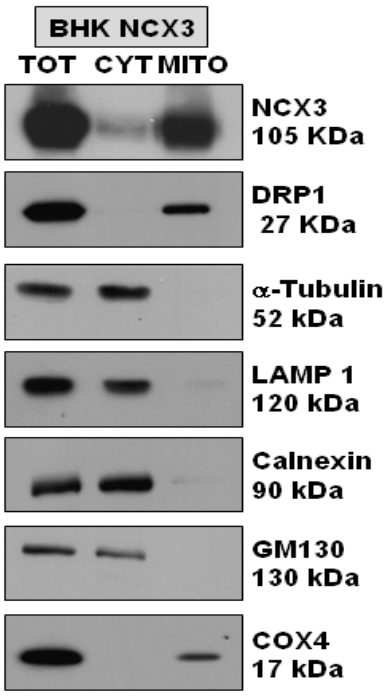

Fig 9: Validation of the purity of mitochondrial preparation. Absence of ER (calnexin), Golgi (GM130) and lysosomes (LAMP1) contamination on mitochondrial fraction (DRP1 and COXIV positive) in BHK-NCX3 stably transfected cells.

Interestingly, confocal double immunofluorescence experiments showed the coexistence of NCX3 immunoreactivity with MitoTracker-stained mitochondria, both along neurites and in the cell body of cortical neurons (Figure 10A, panels d,e). Furthermore, a higher magnification image of a single mitochondrion from neurites (Figure 10A, panel f) revealed that NCX3 immunosignal was selectively localized at the level of mitochondrial membranes. The calculated percentage of NCX3 immunoreactivity in mitochondria corresponds to 25% of the total NCX3 signal (Figure 10B).

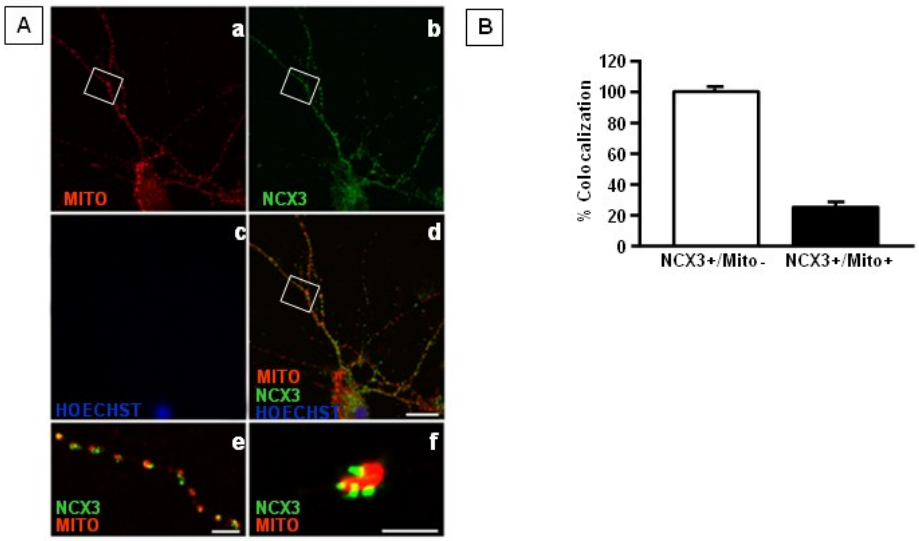

Fig.10: Mitochondrial distribution of NCX3 in cortical neurons. A(panels a-d), A representative cortical neuron double-labelled with NCX3(green) and MitoTracker(red).(**e**), Co-expression of NCX3 punctate staining(green) with mitochondria(red) along a single neurite.(**f**), Higher magnification image depicting NCX3 punctate distribution on a single mitochondrion. Scale bars:20μm in a-d;5μm in e;2μm in f.**(B),** Quantification of co-localization amount of NCX3 immunosignal at mitochondrial level measured as percentage of the total NCX3 immunosignal.

Electron microscopy experiments were performed in NCX3+/+ and NCX3-/- neurons (Figure 11A) demonstrating that surface gold density for NCX3 in mitochondria corresponded to 0.19 AU, in ER corresponded to 0.24 AU and, finally, in plasma membrane corresponds to 0.23 AU in neurons (Figure 11B).

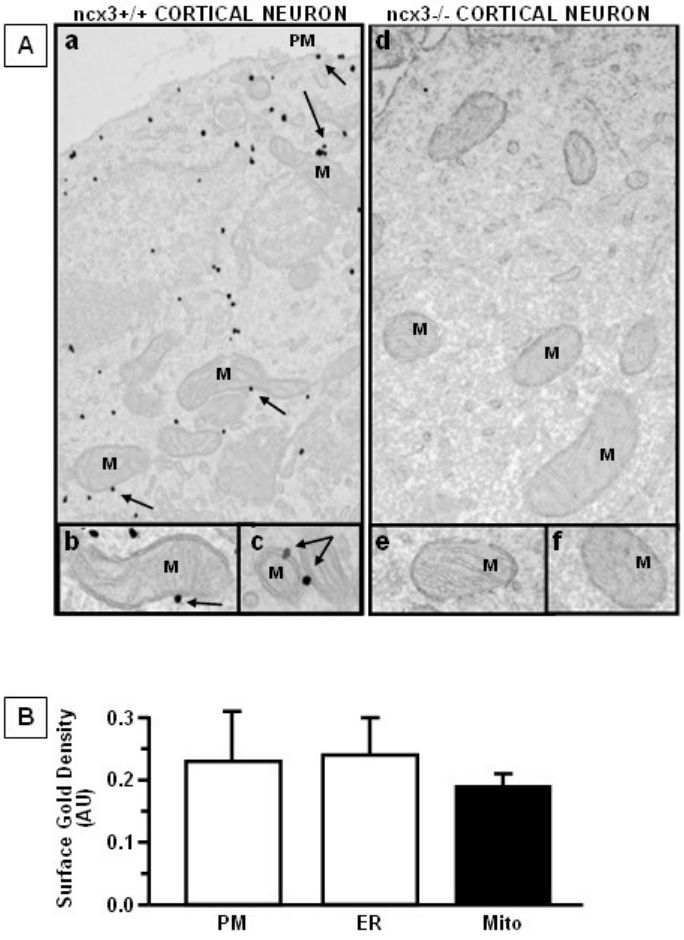

Fig.11: Mitochondrial distribution of NCX3 in cortical neurons. (A) Distribution and **(B)** quantification of endogenous NCX3 in NCX3+/+ and NCX3-/- neurons along the PM, ER and mitochondria(arrows).Insets show NCX3 exclusively on OMM.

These morphological data were corroborated by Western Blot analysis. Indeed, mitochondria obtained from whole brain of NCX3 +/+ mice displayed a clear-cut NCX3 immunoreactivity.

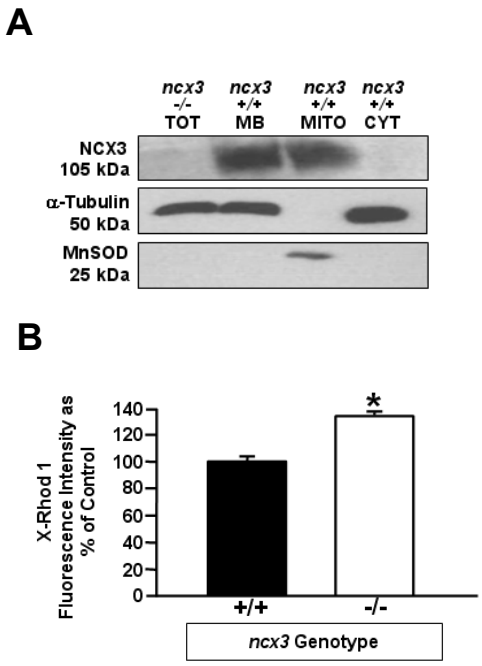

Fig.12: Mitochondrial expression and function of NCX3 in the brain. (A), Localization of NCX3 within membrane, cytosolic and mitochondrial fractions obtained from total mouse brain of wild type NCX3+/+ mice and within total lysates obtained from NCX3-/- mice. **(B)**, Quantification of basal $[Ca^{2+}]_m$ levels measured in cortical neurons obtained from NCX3-/- and NCX3+/+ mice.

Once demonstrated that NCX3 is present on mitochondria, further experiments were performed in order to demonstrate its role in mitochondrial calcium handling. To this aim cells were loaded with the fluorescent probe X-Rhod-1 and $[Ca^{2+}]_m$ measured by a confocal approach. The results of these experiments demonstrated that basal $[Ca^{2+}]_m$ levels were higher in cortical neurons obtained from NCX3-/- than in NCX3+/+neurons (Figure12B). Conversely, BHK cells

over-expressing NCX3 displayed lower $[Ca^{2+}]_m$ than did BHK-Wt cells (Figure 13).

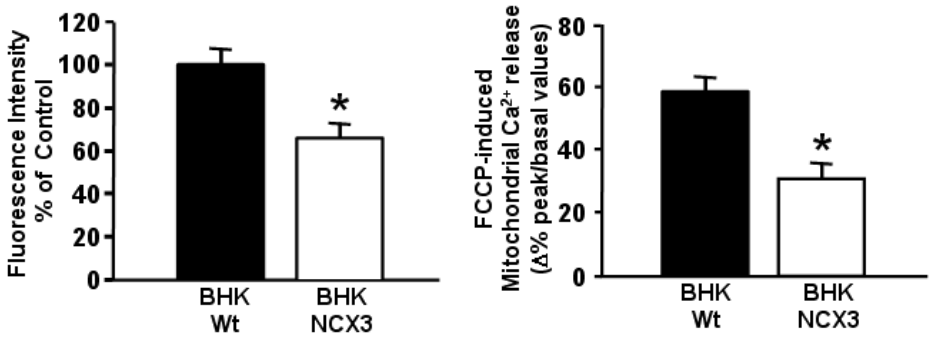

Fig.13: Quantification of basal mitochondrial $[Ca^{2+}]_m$ measured in BHK-Wt and in stably transfected BHK-NCX3 cells. **P*<0.05 vs BHK-Wt cells

4.2 Biochemical interaction between NCX3 and AKAP121 on the outer mitochondrial membrane

Once NCX3 was localized on the OMM, further experiments were performed to characterize its biochemical properties. In particular, we hypothesized whether the PKA anchoring protein AKAP121, which is exclusively localized on the mitochondria, might play a role in the regulation of mitochondrial NCX3 activity. This hypothesis was supported by the observation that NCX family proteins have been demonstrated to possess multiple sites of phosphorylation, by PKA and PKC responsible of regulating Na^+/Ca^{2+} exchanger activity, in response to

both physiological and pathophysiological stimuli (Blaustein and Lederer, 1999; Schulze et al., 2003). To test this hypothesis, BHK-Wt cells were transiently co-transfected with NCX3-Flag ($NCX3^F$) and AKAP121. In these cells, confocal microscopy showed that $NCX3^F$ immunoreactivity co-localized in a large number of mitochondria stained with MitoTracker (Figure 14A, panels a-h). Double immunofluorescence experiments (Fig.14A, panels i-l) performed with both anti-NCX3 and anti-FLAG antibodies revealed the co-existence of NCX3 and $NCX3^F$ immunosignals.

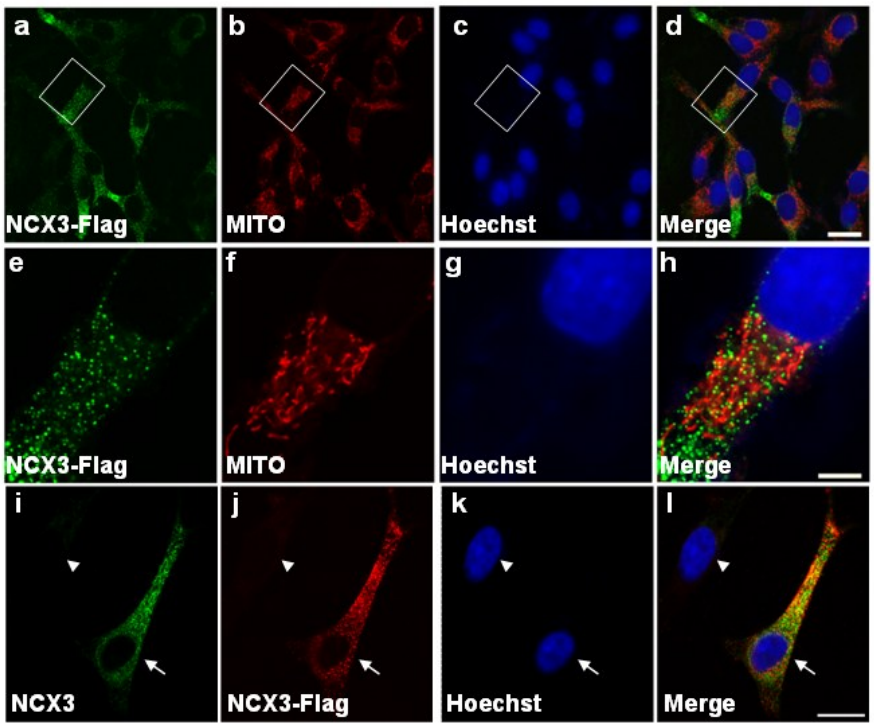

Fig.14: Localization of NCX3^F at mitochondrial level in BHK WT cells. (panels a-d) Co-localization of anti-FLAG antibodies(green) with MitoTracker(red) in BHK-; **(panels e-h)** higher magnification images of the frame depicted in a-d showing numerous NCX3 puncta localized on mitochondria. Scale bars: 20μm in a-d; 5μm in e-h; **(panels i-l)** co-localization of anti-FLAG antibody(red) with NCX3 antibody(green) in BHK-NCX3^F.Scale bars: i-l, 20μm

In addition, immunocitochemistry experiments showed that AKAP121 co-localized with NCX3^F in BHK cells co-transfected with both constructs (Figure 15A). Moreover, we performed co-immunoprecipitation experiments using total lysates prepared from BHK wild type, transiently NCX3-transfected and NCX3+AKAP121 co-transfected cells. Forty-eight hrs following transfection, total lysates were prepared and subjected to immunoprecipitation with anti-Flag antibody. Subsequently, the precipitates were immunoblotted with anti-AKAP121 antibody. The results obtained demonstrated that NCX3 and AKAP121 form a stable complex in transiently NCX3^F+AKAP121 co-transfected BHK cells (Figure 15B). Conversely, in BHK cells singly transfected with NCX3^F, only a light co-precipitated band was observed, implying the possible involvement of the constitutively expressed AKAP121 protein.

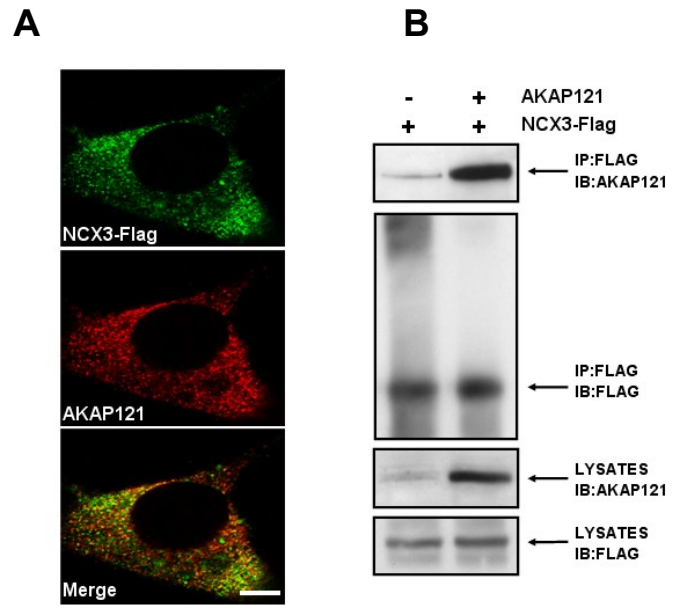

Fig.15: Interaction between NCX3 and AKAP121 in double transiently co-transfected BHK cells. (A) Co-expression of anti-Flag (green) and AKAP121 (red) antibodies in BHK-NCX3^F cells. Scale bar in a-c: 5μm. **(B)** Cells extracts from BHK-AKAP121 and BHK-AKAP121+NCX3^F were subjected to immunoprecipitation with anti-Flag antibodies followed by immunoblotting with either anti-AKAP121 or anti-Flag antibodies.

4.3 Functional interaction between mitochondrial NCX3 and AKAP121

To demonstrate that the molecular interaction between NCX3 and AKAP121 may be responsible for the mitochondrial Ca^{2+} handling, further experiments were performed on BHK cells transiently co-transfected with NCX3^F and AKAP121. In these cells, basal $[Ca^{2+}]_m$ were lower than those measured in single transiently transfected AKAP121, NCX3^F, and Wt BHK cells (Figure 16).

However, $[Ca^{2+}]_m$in BHK-NCX3 transiently transfected cells resulted higher compared to $[Ca^{2+}]_m$ measured in stably transfected BHK-NCX3 cells. This was probably due to an adaptative response occurring in stably transfected cells which affects intracellular calcium homeostasis (Fig.16 vs Fig.13). Interestingly, when the cells were transiently co-transfected with $NCX3^F$ and the $AKAP121_{L313-319P}$, a mutant unable to bind PKA, the $[Ca^{2+}]_m$ were higher than those detected in BHK cells transiently co-transfected with $NCX3^F$ and AKAP121. Similar results occurred when these cells were further transiently co-transfected with the specific Protein Kinase Inhibitor (PKI) construct, which is able to inhibit the catalytic subunit of PKA (Figure 16).

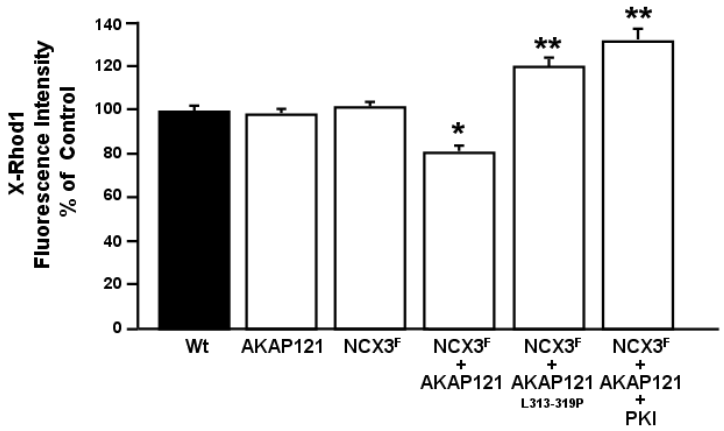

Fig.16: $[Ca^{2+}]_m$ in BHK WT and in BHK-$NCX3^F$ transiently transfected. Basal $[Ca^{2+}]_m$ in BHK-Wt cells and transiently transfected with AKAP121, $NCX3^F$, $NCX3^F$+AKAP121, $NCX3^F$+$AKAP121_{L313-319P}$, and the PKI construct.*P<0.05 vs BHK–Wt cells, BHK-AKAP121 or BHK-$NCX3^F$; **P<0.05 vs BHK-$NCX3^F$+AKAP121.

To evaluate the effect of NCX3 and AKAP121 on Ca^{2+} efflux under conditions of

elevated $[Ca^{2+}]_m$, BHK cells co-transfected with $NCX3^F$ and AKAP121 were first exposed to ionomycin (3μM, 15min) to allow mitochondrial Ca^{2+} loading and to activate mitochondrial Ca^{2+} efflux mechanisms such as mNCX. They were then exposed to the mitochondrial uncoupler FCCP (250nM) to induce mitochondrial depolarization and Ca^{2+} extrusion (Park et al., 2002). After ionomycin treatment, the amount of Ca^{2+} extruded in the cytoplasm upon FCCP exposure was measured as $[Ca^{2+}]_I$ increase. This release is widely considered as an index of mitochondrial Ca^{2+} efflux. For instance, when Ca^{2+} release in the cytoplasm is low, higher activity of mitochondrial Ca^{2+} efflux pathway may occur. Intriguingly, BHK cells transfected only with $NCX3^F$ showed an increase in Ca^{2+} efflux activity as compared to wild type or BHK cells transfected with AKAP121. In these cells, however, Ca^{2+} efflux activity decreased when AKAP121 was silenced but significantly increased when the cells were co-transfected with $NCX3^F$ and AKAP121 (Figure 17). Moreover, when the catalytic subunit of PKA was inhibited by the PKI construct, the effect of AKAP121 was neutralized and the resulted Ca^{2+} efflux was similar to that obtained in $NCX3^F$ transfected cells. The specificity of siRNA for AKAP121 was reported in Figure 17.

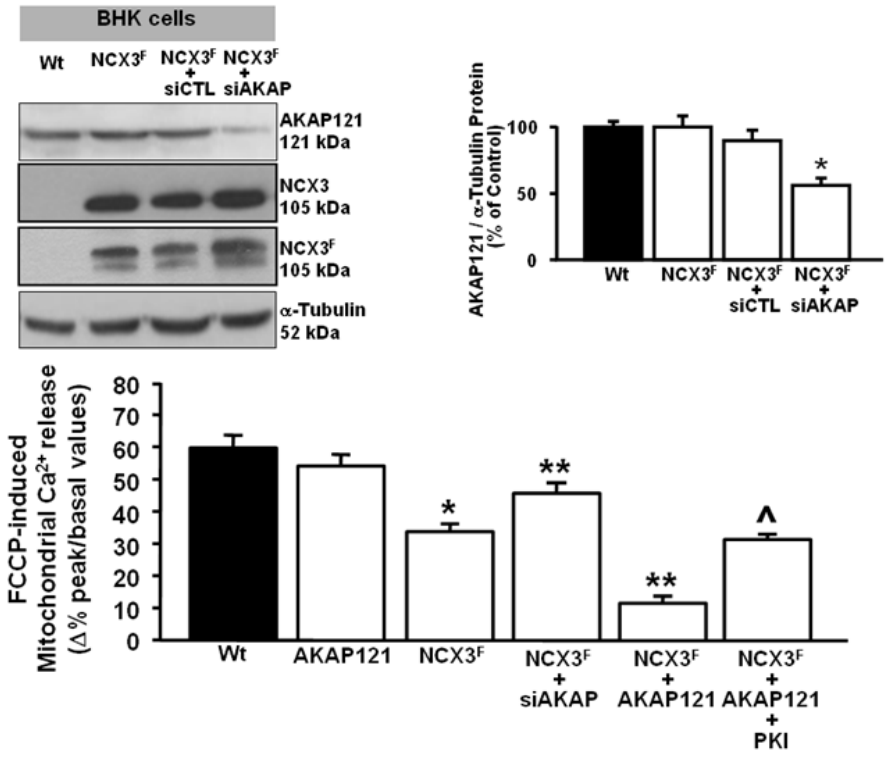

Fig.17: Effect of ionomycin on $[Ca^{2+}]_m$ in BHK-NCX3^F+AKAP121, BHK-NCX3^F+siAKAP121, BHK-NCX3^F+AKAP121+PKI. *P<0.05 vs BHK-Wt and BHK-AKAP121. **P<0.05 vs BHK-NCX3^F;^P<0.05 vs BHK-NCX3^F+AKAP121; **(inset), Effect of siAKAP on AKAP121 protein expression in BHK-Wt and BHK-NCX3^F**. *P<0.05 vs BHK-Wt.

To further demonstrate that mNCX3 controls Ca^{2+} efflux from mitochondria, BHK NCX3^F and NCX3^F+AKAP121 transiently co-transfected cells were preincubated with the putative inhibitor of mitochondrial Na^+/Ca^{2+} exchanger CGP-37157 (Cox et al.,1993) and then exposed to FCCP to induce mitochondrial Ca^{2+} extrusion. CGP-37157 (10μM) reduced FCCP-induced mitochondrial Ca^{2+} extrusion in BHK cells transiently transfected either with NCX3^F or NCX3^F+AKAP121. Conversely,

CGP-37157 was ineffective in BHK–Wt cells since this clone does not express the NCX isoforms (Figure 18). Moreover, to rule out the possibility that ionomycin or CGP-37157 could trigger a depolarization of the mitochondrial membrane, we measured $\Delta\Psi_m$ in ionomycin- and CGP-treated cells (Figure 18 inset).

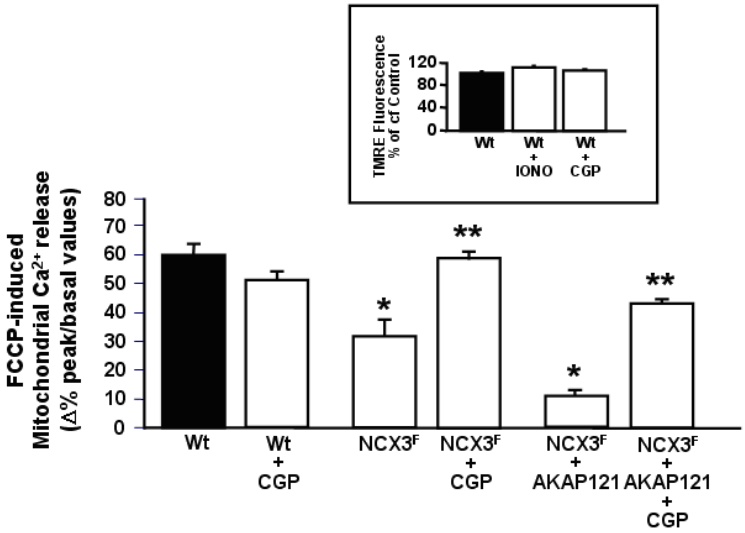

Fig.18: Effect of CGP-37157 on $[Ca^{2+}]_i$ measured in BHK-Wt and BHK-$NCX3^F$ or BHK-$NCX3^F$+AKAP121 transfected cells. **P*<0.05 vs BHK-Wt; ***P*<0.05 vs BHK-$NCX3^F$ and vs BHK-$NCX3^F$+AKAP121; **(inset),** $\Delta\Psi_m$ in BHK-Wt cells after treatment with CGP-37157 and with ionomycin. Each bar represents the mean ± S.E.M. of different experimental values in 3 independent experimental sessions.

4.4 Effect of hypoxia on mNCX3 activity in cortical neurons and in $NCX3^F$-AKAP121 BHK transfected cells

To demonstrate whether endogenous NCX3 modulates mitochondrial Ca^{2+}

extrusion, experiments were performed in cortical neurons exposed to Oxygen and Glucose Deprivation (OGD) followed by Reoxygenation (Rx). Quantitative co-localization analysis of NCX3 with Mitotracker demonstrated that NCX3 mitochondrial immunosignal decreased during OGD and returned to the basal level after OGD/Rx (Figure 19).

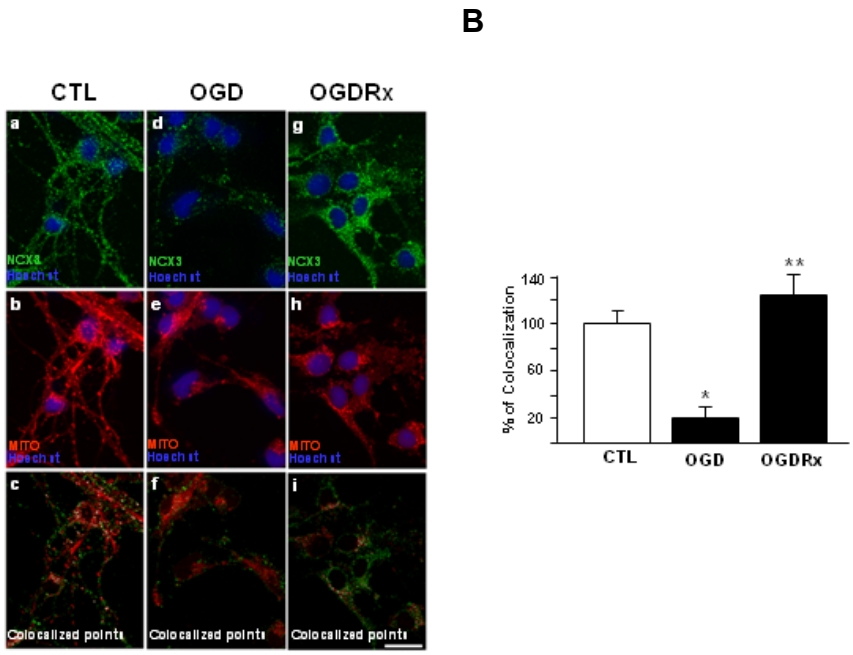

Fig.19. Localization of mNCX3 in cortical neurons exposed to OGD and OGD/Rx. A, Confocal double immunofluorescence images displaying both NCX3 (green) and Mito (red) immunosignals in cortical neurons under control conditions (panels a-c) and following OGD (d-f) or OGD/Rx (g-i). Superimposed images displaying co-localizing pixels (white*)* in panels c, f and i. Scale bars: a-i: 20μm. **B,** Quantification of the number of NCX3/Mito colocalized points (white). Each bar represents the mean ± S.E.M. of the data obtained from 10 microscope fields per group in 3 independent experimental sessions.*P<0.05 vs CTL**;** **P<0.05 vs OGD.

Accordingly, $[Ca^{2+}]_m$ significantly increased when neurons were exposed to

OGD, whereas decreased following OGD/Rx (Figure 20). Interestingly, when NCX3 was knocked down with siRNA, an impairment in mitochondrial Ca^{2+} extrusion was recorded both under basal and OGD/Rx conditions. On the other hand, no alteration occurred in mitochondrial Ca^{2+} extrusion during OGD, a condition in which NCX3 mitochondrial immunosignal decreased.

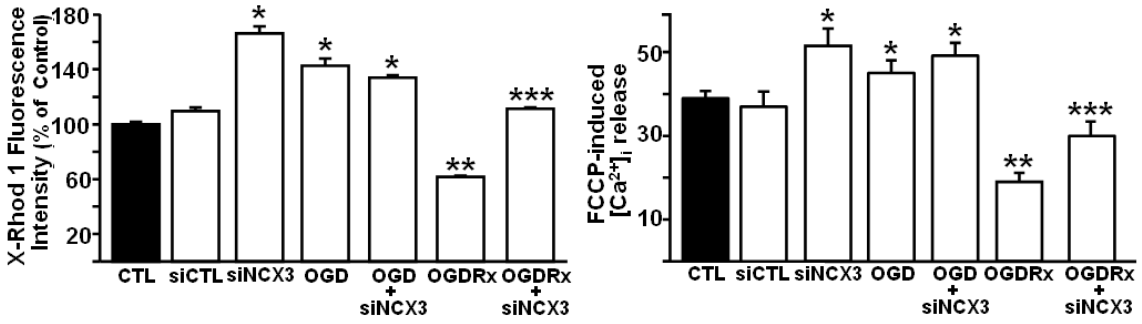

Fig.20: **$[Ca^{2+}]_m$ measured by X-Rhod1 and Fura-2AM in cortical neurons transiently transfected with siNCX3 and exposed to OGD and OGD/Rx**. **P*<0.05 vs CTL;***P*<0.05 vs OGD;****P*<0.05 vs OGD/Rx

The increase in $[Ca^{2+}]_m$ elicited by chemical hypoxia in BHK-Wt cells was reduced when these cells were transfected with $NCX3^F$, thus showing that mNCX3 was working as a Ca^{2+} efflux pathway (Figure 21). Moreover, when BHK cells were transfected with $NCX3^F$ and, subsequently, with $AKAP121_{L313\text{-}319P}$ mutant, $[Ca^{2+}]_m$ increased in response to chemical hypoxia. Similarly, the silencing of endogenous AKAP121 in BHK cells transfected with $NCX3^F$ reduced

Ca^{2+} efflux activity of the mNCX, as demonstrated by the increase in the $[Ca^{2+}]_m$ caused by chemical hypoxia (Figure 21). Interestingly, the transfection of the PKI construct in $NCX3^F$+AKAP121 transiently co-transfected BHK cells prevented the reduction in $[Ca^{2+}]_m$ operated by the exchanger and the anchoring protein during chemical hypoxia (Figure 21).

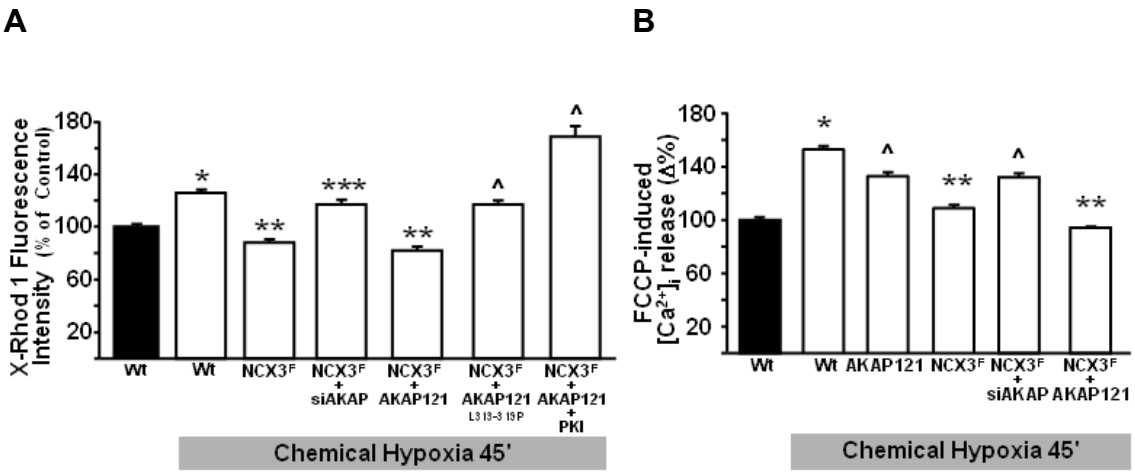

Fig.21. Effect of chemical hypoxia on $[Ca^{2+}]_m$ in NCX3-AKAP-121 BHK transfected cells. $[Ca^{2+}]_m$ measured after 45 min chemical hypoxia exposure in BHK-WT cells and in BHK-WT cells transiently transfected with NCX3-Flag ($NCX3^F$), $NCX3^F$+ siAKAP-121; $NCX3^F$+ AKAP-121, $NCX3^F$+ $AKAP121_{L313\text{-}319P}$ mutant and $NCX3^F$+ AKAP-121+ PKI construct. The quantification of $[Ca^{2+}]_m$ is expressed as arbitrary units of X-Rhod1 fluorescence under normoxic conditions (black bar) and after 45 min of chemical hypoxia (white bars). Each bar represents the mean ±S.E.M. of different experimental values studied in three independent experimental sessions. *P < 0.05 vs.control BHK-WT cells; **P < 0.05 vs. BHK-WT cells exposed to 45 min of chemical hypoxia; ***P < 0.05 vs. BHK-$NCX3^F$ and transfected cells exposed to chemical hypoxia; ^P < 0.05 vs. BHK-$NCX3^F$ + AKAP-121 co-transfected cells exposed to chemical hypoxia.

In agreement with these results, cell survival in response to chemical hypoxia was higher in BHK cells transfected with $NCX3^F$ or co-transfected with

$NCX3^F$+AKAP121. Moreover, the silencing of constitutively expressed AKAP121 completely reverted the pro-survival effect exerted by either $NCX3^F$ or $NCX3^F$+AKAP121 transfection (Figure 22).

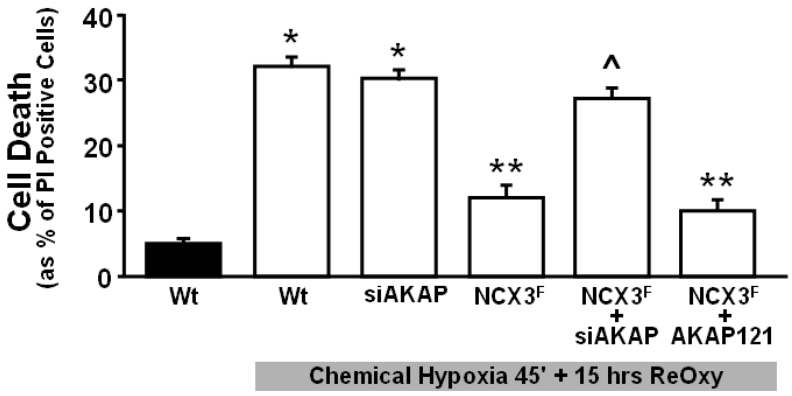

Fig.22 Effect of chemical hypoxia + 15 hours of reoxygenation on cell viability in BHK-WT cells and in BHK transiently transfected with $NCX3^F$, $NCX3^F$ + AKAP121, and $NCX3^F$ + siRNA AKAP-121. The cells were double stained with 36 μM fluorescein diacetate and 7 μM propidium iodide (PI) under control conditions and after 45 min of chemical hypoxia + 15 hours of reoxygenation. *P < 0.05 vs. respective control; **P < 0.05 vs. BHK-WT cells exposed to 45 min chemical hypoxia + 15 h reoxygenation; **P < 0.05 vs. BHK-$NCX3^F$ cells exposed to 45 min chemical hypoxia + 15 h reoxygenation.

4.5 Effect of hypoxia on mitochondrial morphology in primary neurons

Primary cultures of hippocampal neurons (Figure 23) were exposed to a protocol of chemical hypoxia for 45 minutes. After this treatment the cells were incubated with fluorescent probes specific for the mitochondrial mass, such as Mitotracker Red CMXRos or MitoTracker GreenTM. The neurons showed a strong alteration

of mitochondrial morphology after the hypoxic stimulus: from elongated to small and spherical mitochondria.

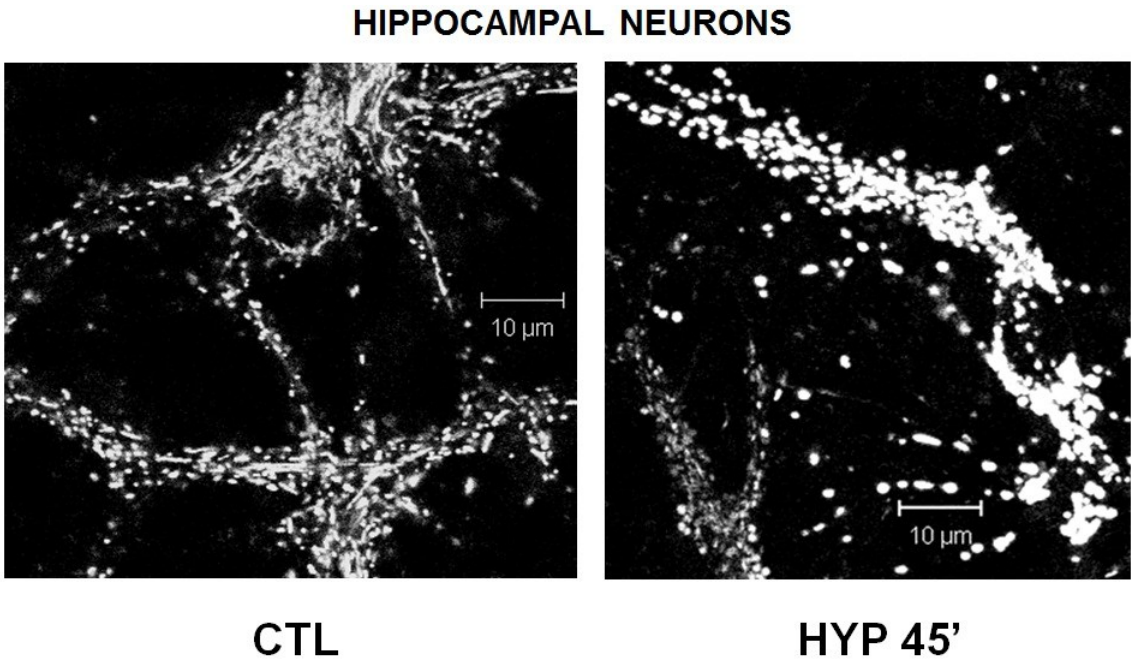

Fig.23: Effect of chemical hypoxia on mitochondrial morphology in hippocampal neurons

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4.6 Analysis of mitochondrial morphology using the *ImageJ 1.42* software

To label mitochondria, the cells were incubated with MitoTracker Red (Invitrogen, 20 nM) for 15 min prior to fixation. Digital images were captured on a confocal microscope, using a 100X 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 (Methods in Enzymology, 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. The values assigned to each element, specifically to each mitochondrion, vary from 0 (circular mitochondria, completely fragmented) to 4 (elongated and highly interconnected).

Primary cultures of hippocampal neurons were exposed to a protocol of chemical hypoxia for 45 minutes, incubated with MitoTracker and, therefore, analysed by confocal microscopy in order to obtain several images to process by the macro "Morphometry". The obtained results showed that in the cells subjected to hypoxic insult, the values of FF and AR of mitochondria were significantly lower than those measured in control cells. (Figure 24).

A **B**

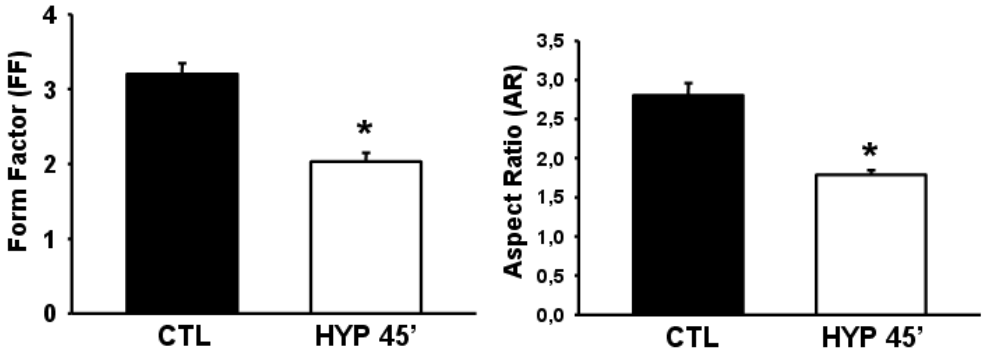

Fig.24: A, FormFactor and B, Aspect Ratio in hippocampal neurons subjected to chemical hypoxia. *P<0.05 vs CTL cells

Similar results were obtained both in hippocampal and cortical neurons exposed to 3 and 6 hours of OGD. After this treatment the cells showed a reduction of the FF and AR values, suggesting a marked effect of hypoxia on the mitochondrial morphology (Figure 25).

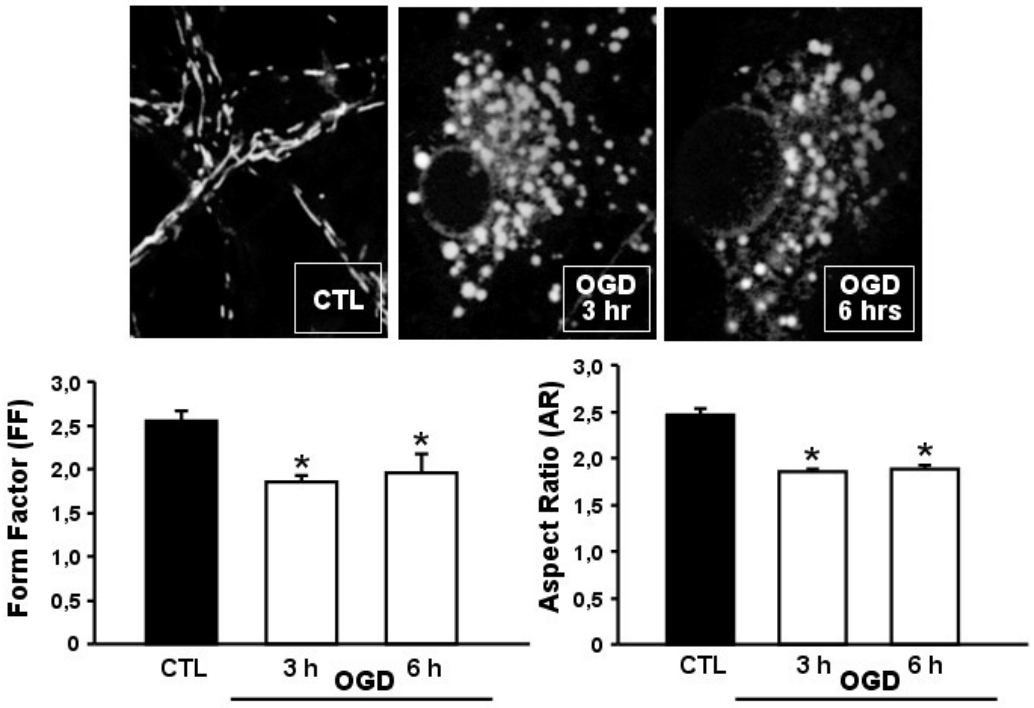

Fig.25: A, FormFactor and B, Aspect Ratio in cortical neurons exposed to 3 and 6 hours of OGD. **P*<0.05 vs CTL cells

4.7 Analysis of DRP-1 and MFN-2 expression in hippocampal neurons exposed to OGD

Further aim of the study was to evaluate changes in DRP-1 and Mfn-2 expression after ischemic insult. The results obtained showed that the levels of DRP-1 expression significantly increased in hippocampal neurons exposed to 3 and 6 hours of OGD, while expression levels of MFN2 decreased, suggesting

the involvement of these proteins in mitochondrial fragmentation event during the ischemic process (Figure 26).

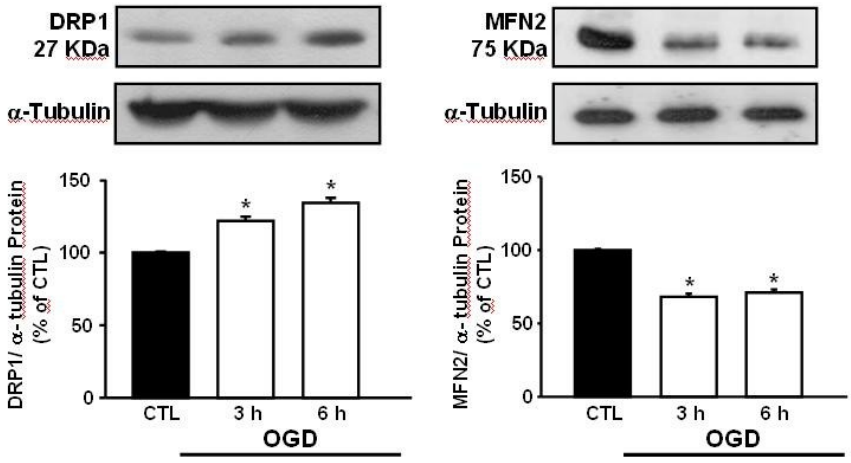

Fig.26: DRP-1 and MFN2 expression in hippocampal neurons exposed to 3 and 6 hours of OGD. *P<0.05 vs CTL cells

4.8 Quantification of mitochondrial functional parameters in cortical neurons exposed to 6 hours of OGD.

Once demonstrated that the neurons exposed to OGD showed mitochondrial morphology changes and alterations of the mitochondrial fusion and fission events, the next step of the study was to verify a relationship between these phenomena and mitochondrial functions. To this aim, confocal microscopy experiments were performed to evaluate the mitochondrial membrane potential in cortical neurons exposed to OGD. The neurons were incubated with TMRE

and the obtained results showed that the intensity of fluorescence measured in mitochondria of neurons exposed both to 3 and to 6 hours of OGD was significantly lower than that measured in mitochondria of control neurons (Figure 27A). In parallel, we measured the intramitochondrial calcium levels with the fluorescent probe X-Rhod1. The results obtained showed an increase in the intensity fluorescence of mitochondria in neurons exposed to OGD (3 and 6 hours) compared to control cells (Figure 27B).

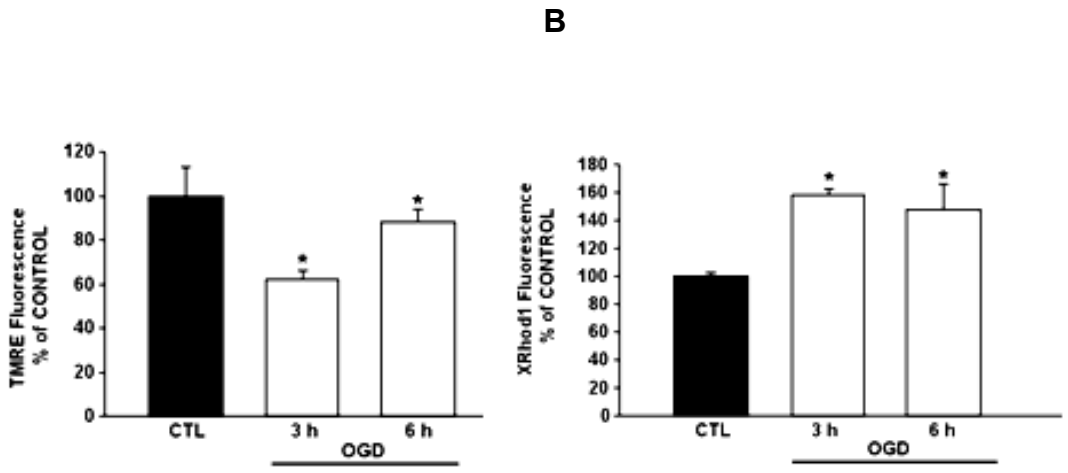

Fig.27: A, Effect of OGD on $\Delta\psi_m$ and B, on $[Ca^{2+}]_m$ in cortical neurons. **P*<0.05 vs CTL cells

Furthermore it has long been known that hypoxia is able to induce a significant depletion of ATP. Indeed, hippocampal neurons exposed to 3 and 6 hours of

OGD showed a significant reduction in ATP levels compared to baseline levels of ATP measured in control cells (Figure 28).

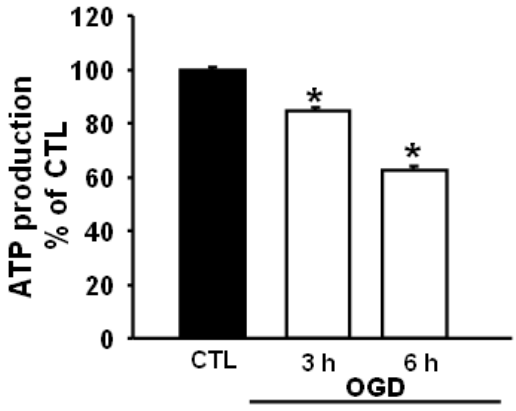

Fig.28: Quantification of ATP content in hippocampal neurons exposed to OGD. **P*<0.05 vs CTL cells

4.9 Effect of OGD on mitochondrial morphology in *Siah2* +/+ versus *Siah2-/-* cortical neurons.

Siah's contribution to mitochondria function was also provided by its ability to regulate the stability of A-kinase anchoring protein 121 (AKAP121) (Carlucci et al., 2008). Control of AKAP121 stability suggests a possible role for Siah2 in regulating mitochondrial membrane potential, mitochondrial activity and possibly oxidative phosphorylation. Given that AKAP121 was demonstrated to be a Siah2 substrate in mitochondria (Carlucci et al., 2008), we assessed the possibility that AKAP121 plays a role in regulating mitochondrial morphology. Indeed, it was

recently shown that AKAP121 is rapidly degraded in cells subjected to hypoxia by a mechanism mediated by the activation of SIAH2. This effect associates with a loss of mitochondrial membrane potential and reduced mitochondrial metabolic activity (Carlucci et al., 2008). Considering these observations we used neurons from *Siah2 +/+* and *Siah2 -/-* mice to evaluate changes of mitochondrial morphology and functional parameters, such as mitochondrial membrane potential, mitochondrial calcium concentration and ATP production. The data obtained showed that the reduction of FF and AR observed during OGD in neurons Wt reverted in the absence of Siah2.

A **B**

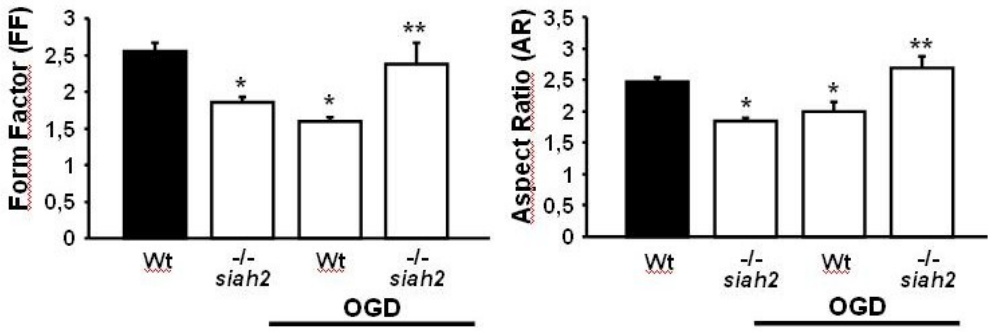

Fig.29: A, FormFactor and B, AspectRatio in cortical neurons obtained from *Siah2 +/+* and *Siah2 -/-* mice. **P*<0.05 vs CTL cells

Once demonstrated that mitochondria of neurons *siah2 -/-* exposed to OGD did not show the same morphological changes observed in mitochondria of the neurons *siah2+/+*, further experiments were performed in these neurons to evaluate differences in mitochondrial functional parameters. The obtained results demonstrated that after OGD the neurons *siah2 -/-* did not show the same alteration of $\Delta\psi_m$ (Figure 30A), the same reduction in $[Ca^{2+}]_m$ (figure 30B) and the same alteration of ATP levels (Figure 30C) observed in neurons *siah2+/+*, suggesting the involvement of AKAP121 protein in the phenomenon of mitochondrial fragmentation during hypoxic stimulus.

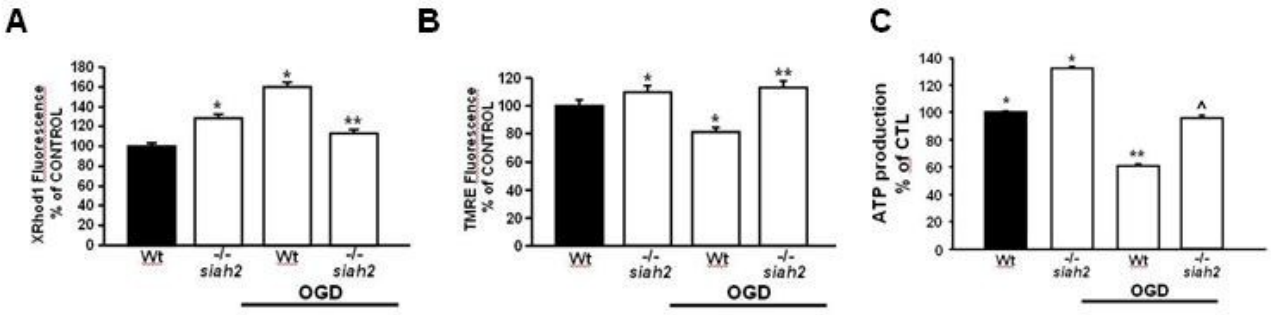

Fig.30: A, Mitochondrial $[Ca^{2+}]$ B, $\Delta\psi_m$, and C, ATP content in cortical neurons *siah2-/-* and *siah2+/+* exposed to 3 hrs of OGD. **P*<0.05 vs Wt cells

Since the previous data demonstrated that mitochondrial calcium homeostasis seems be involved in the alteration of mitochondrial dynamics during the

neuronal ischemic death, the next step was to verify the role of NCX3 and the putative interaction between this protein and AKAP121 in this phenomenon. To this aim the neurons were exposed to OGD, a condition in which NCX3 and AKAP121 expression were reduced, followed by OGD/Rx, a condition in which NCX3 and AKAP121 expression returned to the basal values (Carlucci et al., 2008; Sirabella et al., 2009). Interestingly, after the Reoxygenation the reduction of mitochondrial FF and AR values observed during OGD returned to the basal values (Figure 31). These results might be related to changes in the expression of endogenous NCX3 and AKAP121 during OGD and OGD/Rx, suggesting the involvement of these two proteins in the alteration of mitochondrial morphology observed during hypoxia.

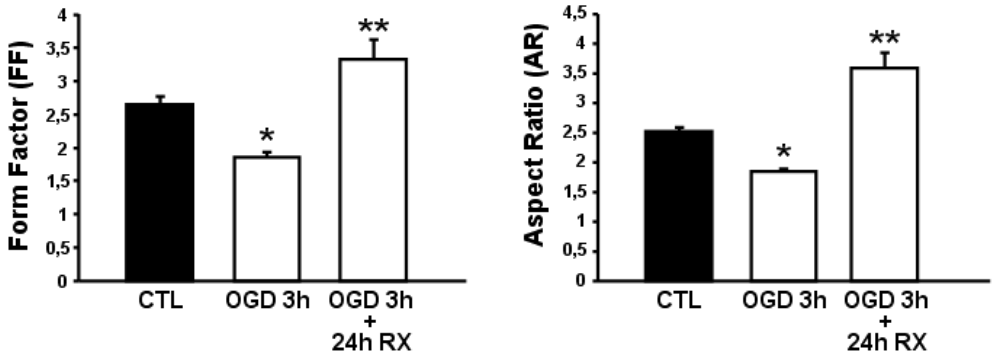

Fig.31: FF and AR in cortical neurons exposed to OGD and OGD/Rx. *P<0.05 vs CTL cells

5.DISCUSSION

The results of the present study clearly demonstrate that the nuclear encoded NCX3 is the only isoform of the Na^+/Ca^{2+}-exchanger localized within the outer mitochondrial membrane (OMM), where it plays a relevant role in the control of mitochondrial Ca^{2+}homeostasis both under basal and under hypoxic conditions.

First, this study provides evidence that only the nuclear encoded NCX3 isoform, but not NCX1 and NCX2, was localized on mitochondria. In particular, mNCX3 was specifically localized on the OMM, as its lysis by detergent agents completely eliminated NCX3 immunoreactivity at mitochondrial level. In this regard, an even more convincing result was that NCX3 gene ablation induced protein loss from the OMM and mitochondrial Ca^{2+}accumulation in cortical neurons. Interestingly, in neurons NCX3-mitochondrial localization is particularly evident along the neurites and in the neuropils close to the plasmatic membrane, where ATP is necessary to drive the activity of those proteins involved in the regulation of ionic homeostasis (Blaustein et al., 2002; Lytton et al., 2009). Recently the group of Palty et al. identified in the inner mitochondrial membrane (IMM), particularly within the cristae, another component of the Na^+/Ca^{2+}exchanger family, namely, NCLX. This Li^+ sensitive protein is both phylogenetically and functionally distinct from NCX and NCKX family members (Palty et al., 2010). These authors also showed that NCLX participates to the

mitochondrial Na^+/Ca^{2+} exchanger activity. This novel finding is not in contrast with the results of this study, in that we proposed a model in which the Na^+/Ca^{2+} exchange activity in mitochondria requires two consecutive steps. The first, operated by the Na^+-sensitive NCLX, mediates Ca^{2+} transport from the matrix to the intermembrane space, and the second one, operated by mNCX3, that promotes Ca^{2+} efflux from the intermembrane space to the cytosol. This interpretation is in line with the recent physiological role attributed to the OMM in the control of mitochondrial Ca^{2+} cycling. Indeed, although the outer surface of the membrane is not a passive permeable membrane, it does constitute a permeability barrier not only to Ca^{2+} influx but also to Ca^{2+} efflux (Szabadkai et al., 2008). On the other hand, evidence that mNCX3 is involved in Ca^{2+} extrusion is that the Ca^{2+} lowering effect found in cells transfected with $NCX3^F$ was completely prevented by the benzothiazepine compound CGP-37157, a selective mNCX inhibitor (Cox et al., 1993; Nicolau et al., 2009).

An interesting finding emerging from our studies is the demonstration that mNCX3 co-localizes with AKAP121, a member of PKA anchoring protein expressed on the OMM, and that this interaction modulates mNCX3 activity. Such finding led to the hypothesis that the interplay between mNCX3 and AKAP121 contributes to cell survival. Indeed, when the constitutively expressed AKAP121 was silenced, the prosurvival effect exerted by the overexpression of

these two proteins was prevented. It was previously demonstrated that AKAP121 regulates the activity of the components of the mitochondrial respiratory chain, thus promoting $\Delta\Psi_m$ hyperpolarization and improving the oxidative synthesis of ATP in a PKA dependent manner (Livigni et al., 2006). Similarly, in this study, immunoprecipitation assays and confocal microscopy experiments revealed that mNCX3 interacts with AKAP121, thus suggesting involvement of the anchoring protein in promoting mNCX3 Ca^{2+} extrusion activity under basal and hypoxic conditions. Consistently, when BHK cells either transfected with NCX3 or co-transfected with NCX3+AKAP121 were treated with CGP-37157, $[Ca^{2+}]_m$ was higher than the one observed in untreated cells. This finding thus suggested that either endogenous or overexpressed AKAP121 might play a role in the regulation of mNCX3 efflux activity. Noticeably, these effects seem to be mediated by the anchoring activity of AKAP121 to PKA on mitochondria, since the kinase inhibition with a specific cDNA construct abolished the AKAP121 effects on $[Ca^{2+}]_m$. This interaction appears to be even more relevant when considering its role in preventing hypoxic cell death. Indeed, the silencing of AKAP121 significantly reduced mNCX3 Ca^{2+} efflux activity and, consequently, increased cell death during hypoxia. Accordingly, we reasoned that the activation of mNCX3 by AKAP121-anchored PKA on the OMM regulates mitochondrial Ca^{2+} handling, thus boosting mitochondrial metabolism and, in turn

cell survival. These results are in line with previous data obtained in our laboratory demonstrating that in BHK cells, NCX3 isoform significantly contributes to the maintenance of $[Ca^{2+}]_i$ homeostasis during experimental conditions mimicking ischemia, thereby preventing $\Delta\Psi_m$ collapse and cell death (Secondo et al., 2007). Moreover, the results obtained in neurons exposed to OGD and OGD/Rx demonstrated that also the endogenous mNCX3 plays a relevant role in the regulation of mitochondrial Ca^{2+} extrusion. Indeed, $[Ca^{2+}]_m$ significantly increased when neurons were exposed to OGD, a condition in which NCX3 expression was reduced. On the other hand, $[Ca^{2+}]_m$ decreased following OGD/Rx, a condition in which NCX3 expression returned to the basal values. Interestingly, when NCX3 was knocked down, an impairment in mitochondrial Ca^{2+} extrusion was recorded both under basal and OGD/Rx conditions, whereas no alteration in mitochondrial Ca^{2+} extrusion occurred during OGD. These results might be related to changes in the expression of endogenous NCX3 and AKAP121 during OGD and OGD/Rx (Sirabella et al., 2009; Carlucci et al., 2008). The identification of NCX3 isoform as a molecular target of PKA on the OMM represents the first evidence for a functional relationship between AKAP121 and those mitochondrial proteins able to regulate mitochondrial Ca^{2+} efflux.

This finding might have important physiological and patho-physiological

implications. Specifically, a considerable crosstalk between bioenergetic function and Ca^{2+} homeostasis occurs within the mitochondria, for Ca^{2+} is necessary to activate mitochondrial oxidative metabolism and to promote mitochondrial respiration (Denton et al., 1980; McCormack et al., 1990; Denton et al., 2009). However, if $[Ca^{2+}]_m$ increases over its buffering capacity, ATP production will decrease, causing mPTP to open, and, eventually, cells to die by apoptosis (Krieger et al., 2002; Jeong et al., 2008). Therefore, this study proposes a model in which mNCX3 complexes with AKAP121 on the OMM and controls mitochondrial Ca^{2+}efflux and cell survival in a PKA-sensitive manner, suggesting that the identification of the mitochondrial complex mNCX3/AKAP121 is able to finely tune mitochondrial calcium handling from the OMM. This evidence might represent an interesting molecular target for the investigation of those mitochondrial dysfunctions involved in neurodegenerative diseases. 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.

In the second part of this study it was demonstrated that in ischemic neurons increase in the expression of Drp1, a protein involved in mitochondrial fission, occurred. This was associated to changes in mitochondrial morphology, mitochondrial membrane potential, mitochondrial calcium concentration and

ATP production. These effects might be related to the reduction in AKAP121 and NCX3 expression occurring during OGD. This hypothesis was supported by the experiments performed in SIAH2 KO mice in which AKAP121 was not degraded during hypoxia. Indeed neurons obtained by SIAH2 KO mice react to hypoxia maintaining high level of ATP and calcium content similar to that observed in WT cells. The obtained results propose a new role for Siah2, in addition to previously characterized Siah2-mediated enhancement of transcriptional reprogramming via HIF-α and inhibition of oxidative phosphorylation by AKAP121 degradation (Nakayama et al., 2004; Carlucci et al., 2008). Indeed it is possible to speculate that Siah2, by regulating AKAP121 on mitochondria could affect mitochondrial Na^{+}/Ca^{2+}exchanger (mNCX3) activity and indirectly play a role in the mechanisms that regulate the mitochondrial metabolic activity during cellular response to hypoxia.

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