Characterization of SCaMC-3, the mitochondrial ATP-Mg/Pi carrier present in liver and brain

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ABBREVIATIONS

AGC: aspartate/glutamate carrier
AMP/ADP/ATP: adenosine mono/di/tri-phosphate
ANT: adenine nucleotide translocator
β-Gal: beta galactosidase
BSA: bovine serum albumin
CaMCs: calcium-binding mitochondrial carriers
CoA: coenzyme A
CRC: calcium retention capacity
CTLN2: adult-onset type II citrullinemia
CypD: cyclophilin D
DIV: Days in vitro
DMEM: Dulbecco's modified Eagle medium
DNA: deoxyribonucleic acid
DTT: dithiothreitol
EDTA: ethylene diamine tetraacetic acid
EGTA: ethylen glycol tetraacetic acid
ER: endoplasmic reticulum
FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone
HPLC: high performance liquid chromatography
IP3: inositol 1,4,5-triphosphate
KA: kainic acid
Km: Michaelis-Menten constant
kDa: kiloDalton
MAS: malate/aspartate shuttle
MCs: mitochondrial carriers
MCAo: middle cerebral arterial occlusion
MCU: mitochondrial calcium uniporter
MSK: mannitol/sucrose/potassium buffer
NAA: N-acetylaspartate
NAD+/NADH: nicotinamide adenine dinucleotide oxidised/reduced
nm: nanometers
OGD: oxygen and glucose deprivation
PBS: phosphate buffered saline
PCR: polymerase chain reaction
Pi: inorganic phosphate
PLC: phospholipase C
PT: permeability transition
PTP: permeability transition pore
PFA: paraformaldehyde
RCR: respiratory control ratio
RNA: ribonucleic acid
RyR: ryanodine receptors
SDS: sodium dodecyl sulphate
S.E.M.: standard error of the mean
SCaMCs: short calcium-binding mitochondrial carriers
TBS: Tris-buffered saline
TNB/DTNB: thionitrobenzoic/dithionitrobenzoic acid
TCA: tricarboxylic acid
X-Gal: bromo-chloro-indolyl-galactopyranoside
SUMMARY

The ATP-Mg/Pi carrier has been described as a putative regulator of gluconeogenesis and the urea cycle, as well as being responsible for the uptake of nucleotides that takes place after birth in newborn liver mitochondria. In addition, recent work has implicated the carrier in mitochondrial calcium homoeostasis and as a regulator of susceptibility to calcium-overload stimuli. In the present work we have studied the function of SCAcM-3, the paralog of the mitochondrial ATP-Mg/Pi carrier found in brain and liver, through the characterization of a mouse model deficient for the protein. Our results indicate that absence of the protein increases the level of liver injury markers in blood and decreases the plasma concentration of urea. In addition, we show that SCAcM-3 can favour coupling between electron transport chain activity and oxidative phosphorylation in adenine nucleotide-depleted mitochondria, a situation similar to the one observed in newborn liver mitochondria, through a mechanism dependent on extramitochondrial calcium that does not require calcium entry in the mitochondria. We have also described that SCAcM-3 is a target of glucagon signalling, being responsible for the uptake of nucleotides by liver mitochondria and the stimulation of respiration observed after its administration. Finally, we show that lack of SCAcM-3 renders mice more susceptible to kainic acid-induced seizures and astrogliosis, but more resistant to ischemia/reperfusion injury both in vivo, with smaller infarct volume after middle cerebral arterial occlusion, and in vitro, with reduced cell death after oxygen and glucose deprivation.
INTRODUCTION
1. THE MITOCHONDRION

Mitochondria are double-membraned organelles present in eukaryotic cells, where they play an important role in generating energy in the form of ATP, as well as regulating processes such as apoptosis, calcium homeostasis and cell proliferation (Nunnari and Suomalainen, 2012). Mitochondria also generate metabolic intermediates that are involved in different pathways, including amino acid metabolism and synthesis of iron-sulphur clusters.

It is now widely accepted that mitochondria evolved from aerobic α-proteobacteria as a result of a process of endosymbiosis (Sagan, 1967), and their origin is linked to the emergence of the eukaryotic cell, which took place ~1.6-2 billion years ago, at the transition from a reducing to an oxidative atmosphere (Knoll et al, 2006). It is thought that the toxicity of oxygen propelled the association of an anaerobic host and an aerobic endosymbiont that evolved to a mitochondrion, reducing this way local oxygen tension (Andersson et al, 2003). As a major evidence for the endosymbiotic theory, mitochondria possess genetic material, which is circular and has a reduced number of genes due to gene transfer to the nuclear genome, ranging 2 (Plasmodium falciparum) to 67 (Reclinomonas americana), most of which code for proteins involved in aerobic respiration and mitochondrial translation (Andersson et al, 2003; Gray et al, 1999). Eukaryotic organisms lacking mitochondria have been identified, but in all cases it has been shown that its absence is due to a subsequent loss or reduction of the organelle (Hrdý et al, 2004; Van der Giezen and Tovar, 2005; Van der Giezen et al, 2002). Two different mitochondrially-derived organelles have been described, mitosomes and hydrogenosomes, both of which have lost their genetic material and most of the oxidative phosphorylation enzymes (Boxma et al, 2005; Dyall and Johnson, 2000). Hydrogenosomes have been detected in the ciliate Nycotherus ovalis, the fungus Neocallimastix frontalis, the flagellate Psalteriomonas lanterna and the human pathogens Trichomonas vaginalis and Tritrichomonas foetus, and are involved in the anaerobic synthesis of ATP by substrate-level phosphorylation using hydrogen as final electron acceptor, (Shiflett and Johnson, 2010). Recently, hydrogenosome-like organelles have also been observed in three newly identified species from the Loricifera phylum, constituting the first anaerobic metazoans described to date (Danovaro et al, 2010). On the other hand, mitosomes are found in the flagellate Giardia lamblia, the intestinal parasite Entamoeba histolytica and in some species of Microsporidia, and do not appear to have any role in ATP synthesis, but rather be exclusively devoted to iron-sulphur cluster biosynthesis (Tovar et al, 2003), an indispensable cellular function that has been proposed to be the common feature of all mitochondrial-related organelles (Lill and Kispal, 2000; Shiflett and Johnson, 2010). Consistently with its lower number of functions, the proteome of mitosomes has been found to be much more reduced than that of mitochondria and hydrogenosomes (Jedelský et al, 2011; Schneider et al, 2011).

In addition to reduced genetic material, a major difference between the free-living α-proteobacteria and the
endosymbiont mitochondria is the presence of mitochondrial carrier proteins, which allow the exchange of solutes and, therefore, its membrane insertion appears to have been an important step in the endosymbiont enslavement process (Cavalier-Smith, 2006).

2. MITOCHONDRIAL CARRIERS
The inner mitochondrial membrane is relatively impermeable and requires the presence of specific proteins to catalyze the exchange of solutes between the cytoplasm and the mitochondrial matrix (Del Arco and Satrústegui, 2005; Kunji, 2004; Palmieri, 2004) (Figure 1.1). These proteins, termed mitochondrial carriers (MCs) and grouped in the SLC25 family, were originated by tandem duplications from a common ancestor and are characterized by the presence of three repeats of about 100 amino acids, each including two transmembrane domains bound by a long hydrophilic loop, and a conserved characteristic sequence (PROSITE: PS50920): Px(D/E)xh(K/R)x(R/K)x20–30(D/E)Gx4a(K/R)GRG, where “X” is any amino acid, “h” an hydrophobic amino acid and “a” an aromatic amino acid (Indiveri et al, 1997).

In yeast genome 34 putative MCs with 27 functionally different transports have been identified, whereas in humans 50 members have been detected, many of which do not have an assigned transport activity (Del Arco and Satrústegui, 2005; Palmieri et al, 2006). It was believed that MCs were exclusively found in

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**Figure 1.1. The family of mitochondrial carriers.** Schematic representation of the inner mitochondrial membrane harboring all mitochondrial carriers. Some metabolic pathways in which the carriers are involved are also depicted. Modified from Palmieri, 2008.
eukaryotes, although recently an MC with unidirectional ATP transport has been described in *Legionella pneumophila*, where it probably arose by horizontal gene transfer (Dolezal et al, 2012). According to the substrates they transport, MCs can be subdivided into four subfamilies which also share sequence similarity: 1) nucleotides and dinucleotides, 2) keto acids, 3) amino acids and 4) other substrates (Robinson and Kunji, 2006). The first of these subfamilies, and in particular the carriers that transport adenine nucleotides, constitute the most complex subgroup of MCs (Del Arco and Satrústegui, 2005; Satrústegui et al, 2007b; Traba et al, 2011).

Defects in MCs are associated with several pathological situations, such as amish microcephaly (mutations in SLC25A19 gene, tiamine pyrophosphate carrier), HHH syndrome (mutations in SLC25A15 gene, ornithine/citrulline carrier) or neonatal myoclonic epilepsy (mutations in SLC25A22 gene, glutamate carrier 1) (Palmieri, 2008).

### 2.1 Structure and transport mechanism

Most MCs catalyse a 1:1 antiport transport, although some carriers can also mediate unidirectional transport (i.e. carnitine/acylcarnitine and some forms of the ATP/ADP carrier) and some catalyse a symport transport with H⁺ (i.e. phosphate, glutamate and pyruvate carriers) (Palmieri, 2004). Until recently, it was widely accepted, that MCs function as homo-dimers, a view that is supported by strong experimental results and that explains how relatively small proteins, with only six helices, can transport large molecules (Kotaria et al, 1999; Schroers et al, 1998). However, X-ray crystallography of the bovine adenine nucleotide transporter (ANT) bound to the inhibitor carboxyatrtyloside indicates that the protein forms a ring-like structure, with a threefold pseudo-symmetry in line with the three-fold sequence repeats, and lacks dimerization sites (Pebay-Peyroula et al, 2003). The analysis of this structure has prompted Kunji and collaborators to propose a new model, where MCs would act as monomers (Kunji and Crichton, 2010). In support for this view, their results show that yeast ATP/ADP carriers function as monomers in mild detergents (Bamber et al, 2007), and a two-dimensional electron microscopy study of the structure of atracyloside-bound yeast ADP/ATP carrier performed by the same group, also indicates a possible monomer functional conformation (Kunji and Harding, 2003).

The sequence similarities between all the members of the MC family suggest that they share a common transport mechanism. According to Kunji's model, two groups of conserved residues are found in the repeated sequences of all MCs and are thought to play an important role: one is the sequence PX[DE]XX[RK], located in the odd helices, on the matrix side of the protein, while the other one is [FY][DE]XX[RK], found on the pair helices, next to the intermembrane space. These sequences form salt bridges between them, and the formation and disruption of these bounds is thought to mediate the transport of the substrates (Figure 1.2) (Robinson et al, 2008).
2.2 Calcium-binding mitochondrial carriers

A subgroup of MCs is characterized by the presence in their N-terminal of EF-hand calcium binding-domains oriented towards the intermembrane space, which renders them susceptible of being regulated by cytosolic calcium (Satrústegui et al, 2007b) (Figure 1.3). These calcium-binding mitochondrial carriers (CaMCs) are the aspartate/glutamate carriers, or AGCs, and the ATP-Mg/Pi carriers, termed SCaMCs for short calcium-binding mitochondrial carriers. AGCs harbour four pairs of EF-hand domains plus a single vestigial EF-hand (Contreras et al, 2007), whereas SCaMCs posses only four EF-hand motifs which, unlike those of AGCs, show high similarity to calmodulin (del Arco and Satrústegui, 2004). The transport activities of both proteins were described decades ago, but the molecular features of the proteins involved have only been recently characterized (Del Arco and Satrústegui, 1998; Del Arco and Satrústegui, 2004; Del Arco et al, 2000; Fiermonte et al, 2004). Last year, a new member of the CaMC
group has been described in the chloroplast membrane, containing a single EF-hand and suggested to catalyse the import of S-adenosyl-methionine (Bayer et al, 2011; Stael et al, 2011).

### 2.2.1. Aspartate/glutamate carriers

The AGCs catalyse the electrogenic exchange of a molecule of aspartate and a molecule of glutamate plus a proton. This activity is irreversible and is the regulatory step of the malate/aspartate shuttle (MAS), that introduces cytosolic NADH into the mitochondria. The basis of this transport were established forty years ago by the group of Katherine LaNoue (LaNoue and Tischler, 1974; LaNoue and Williamson, 1971).

In mammals there are two different paralogs of the AGC, each one with a different pattern of expression in adults: aralar, or AGC1 (SLC25A12), is the most abundant form present in excitable tissues, namely brain and skeletal muscle (Del Arco and Satrústegui, 1998; Palmieri et al, 2001), while citrin, or AGC2 (SLC25A13), is expressed mainly in liver and kidney (Del Arco et al, 2000). Both proteins are co-expressed in heart (Del Arco and Satrústegui, 1998; Del Arco et al, 2000). In yeast, there is only one AGC, Agc1p, which lacks EF-hand domains and can catalyse, in addition to aspartate/glutamate exchange, the uniport of glutamate and, to a lesser extent, of aspartate (Cavero et al, 2003). In humans, mutations in the citrin gene are associated with adult-onset type II citrullinemia (CTLN2) (Kobayashi et al, 1999), while deficiency in aralar is rare and only one patient has been identified to this date (Wibom et al, 2009).

Skeletal muscle and brain mitochondria from mice deficient in aralar show reduced respiratory rates using glutamate and malate as substrates, and no detectable MAS activity (Jalil et al, 2005). In addition, aralar is...
required in the brain for myelin synthesis (Satrústegui et al, 2007a; Satrústegui et al, 2007b). Mice deficient in aralar show defects on myelination soon after birth, and their brain levels of aspartate and N-acetylaspartate (NAA), both of which are intermediates in the synthesis of myelin, are drastically reduced (Jalil et al, 2005; Sakurai et al, 2010). To explain these features, a model has been proposed whereby neuronal mitochondria produce aspartate, which is transported to the cytoplasm through aralar and converted to NAA by the enzyme aspartate-N-acetyl-transferase. NAA is then transaxonally transported to oligodendrocytes, where it supplies acetyl groups for the synthesis of myelin lipids (Satrústegui et al, 2007b). The reason why the onset of myelination defects in aralar<sup>−/−</sup> mice does not occur during early development is probably due to overlapping expression of citrin and aralar on prenatal stages (Del Arco et al, 2002) as well as increased permeability of the blood-brain barrier, which is fully consolidated only after birth (Ramos et al, 2003). At this time, the brain becomes strictly dependent on endogenous production of aspartate. Myelination defects are also observed in the only aralar-deficient patient described to date, which also displays severe muscular hypotonia and reduced muscle mitochondrial respiration with glutamate and malate (Wibom et al, 2009).

Moreover, deficient aralar activity could also be implicated in two other pathological conditions: the Mohr-Tranebjaerg syndrome and autism disorder. The Mohr-Tranebjaerg syndrome is characterized by a defective import of proteins in the mitochondrial inner membrane through the complex DDP1/TIMM8a-TIM13 (Koehler et al, 1999; Roesch et al, 2002), and it has been shown that the AGCs are imported through this pathway (Roesch et al, 2004). Regarding autism disorder, two single nucleotide polymorphisms in the gene that encodes aralar have been reported to be linked with some forms of autism (Ramoz et al, 2004; Segurado et al, 2005; Silverman et al, 2008; Turunen et al, 2008), although another groups have failed to observe this link (Blasi et al, 2006; Correia et al, 2006; Rabionet et al, 2006) (reviewed in Rossignol and Frye, 2012). A recent study using post-mortem tissue from human patients has also connected aralar with autism, though in this case the authors state that the differences observed are a consequence of increased aralar activity due to abnormally high neocortical calcium levels and not to genetic mutations or polymorphisms in the SLC25A12 locus (Palmieri et al, 2010).

On the other hand, mutations in AGC2 underlie CTLN2, an adult-onset liver disease mainly found among the Japanese population, caused by defective activity of argininosuccinate synthase and clinically characterized by hyperammonemia and elevated serum citrulline levels (Kobayashi et al, 1999). Citrin deficiency has been suggested to affect urea synthesis by reducing the efflux of aspartate from the mitochondria to the cytoplasm, where is required to form argininosuccinate from citrulline (Palmieri et al, 2001). In addition, as in the case of aralar, lack of citrin results in deficient NADH import into the mitochondria, which might promote the activity of other NADH shuttles, namely the glycerol-phosphate shuttle and the citrate/malate shuttle (Saheki and Kobayashi, 2002). In the latter case, the citrate/malate
shuttle consumes cytosolic NADH, but produces acetyl-CoA in the cytoplasm, where is used in fatty acid synthesis, which would explain hyperlipidemia and fatty acid liver observed in CTLN2 patients (Saheki et al, 2004).

A different disease termed neonatal intrahepatic cholestasis has also been found in a number of neonates carrying the same mutations in citrin found in CTLN2 patients (Ohura et al, 2001; Tomomasa et al, 2001). Mice deficient in citrin show a phenotype consistent with a defect in MAS, namely reduced gluconeogenesis from lactate, but not from pyruvate, and reduced urea production, but do not exhibit the pathological features from CTLN2 (Sinasac et al, 2004).

2.2.2 ATP-Mg/Pi carriers. The ATP-Mg/Pi carrier catalyses the exchange of inorganic phosphate (HPO$_4^{2-}$) against ATP-Mg$^2+$ or HADP$^2$. This activity is insensitive to inhibitors of the ANT carboxyatractyloside and bonkrekcic acid (Austin and Aprille, 1984), is strictly dependent on micromolar concentrations of calcium (Nosek et al, 1990) and occurs in a non-electrogenic fashion, being therefore reversible and regulated by the concentration gradients of the substrates (Joyal and Aprille, 1992) (Figure 1.4A). The transport activity of the ATP-Mg/Pi carrier was first described by Sutton and Pollak in the late 70s (Sutton and Pollak, 1978) and characterized in detail by Aprille and collaborators in the next two decades (Asimakis and Aprille, 1980b; Austin and Aprille, 1984; Dransfield and Aprille, 1994; Joyal and Aprille, 1992; Joyal et al, 1995; Nosek et al, 1990). When activated, the carrier alters the net adenine nucleotide content of the mitochondria, regulating mitochondrial reactions and pathways with adenine nucleotide-dependent steps (Aprille, 1988) (Figure 1.4B). Although its main substrate is ATP bound to magnesium, the carrier can also exchange free ADP and, to a lesser extent, free AMP (Asimakis and Aprille, 1980b). Due to the relative slower activity of the ATP-Mg/Pi carrier, compared to the ATP synthase and the ANT, Aprille and collaborators stated that its activity does not affect the ATP/ADP ratio in the different cell compartments, but rather only alters the net adenine nucleotide content (Aprille, 1993), although Schild et al have observed a positive correlation between the ATP/ADP ratio and the adenine nucleotide pool size at intermediate rates of respiration (Schild et al, 1999).

The first step towards the molecular characterization of the ATP-Mg/Pi carrier came from the identification of a calcium-binding mitochondrial carrier termed Effinal, and described by immunoelectron microscopy to be localized in peroxisomes and mitochondria from small intestine in rabbit (Weber et al, 1997). Some years later, the genes and proteins responsible for the mitochondrial ATP-Mg/Pi transport activity in humans were unambiguously identified as short calcium-binding mitochondrial carriers or SCaMCs (Amigo et al, 2012; Del Arco and Satrústegui, 2004; Fiermonte et al, 2004; Haitina et al, 2006; Traba et al, 2009b). This group of proteins shows great complexity, including, in humans, five different paralogs with several splicing variants (Satrústegui et al, 2007b) (Figure 1.5): SCaMC-1 (SLC25A24, also
Figure 1.4. The ATP-Mg/Pi carrier. A. Schematic representation of the activity of the ATP-Mg/Pi carrier. Unlike the ATP/ADP carrier, the ATP-Mg/Pi carrier changes the net mitochondrial adenine nucleotide content. The direction of the transport is regulated by the concentration of the substrates, ATP-Mg (or ADP) and phosphate. As phosphate uptake through the phosphate carrier is a very rapid process and is dependent on the proton gradient between the two compartments, the activity of the ATP-Mg/Pi carrier is indirectly regulated by the energetic state of the mitochondria. Adenine nucleotides transported do not alter the ATP/ADP ratio, only affecting the net mitochondrial adenine nucleotide content. B. Representation of adenine nucleotide-dependent reactions that take place in the mitochondrial matrix. These include the first steps of gluconeogenesis and ureogenesis, protein import, intramitochondrial protein synthesis and oxidative phosphorylation.

termed APC1) and SCA3-2 (SLC25A25, also termed APC3), which show different isoforms that are generated by the use of alternative promoters (del Arco and Sattrústegui, 2004); SCA3-3 (SLC25A23, also termed APC2), with multiple splicing variants that differ in their C-terminal (Del Arco, 2005); SCA3-3L (SLC25A41) which has lost the the EF-hand domains is not regulated by calcium (Traba et al, 2009b); and SCA3-1L, the most recently identified member, found as a pseudogene in humans and as an active gene in non-primate mammals, with a protein expression in rodents restricted to testis, where it displays several subcellular locations throughout spermatid differentiation (Amigo et al, 2012). In baker's yeast there is only one paralog, termed Saa1p, which stands for “suppressor of AAC2 lethality”.

Interestingly, it has been suggested that the ATP-Mg/Pi carrier, could have been involved at an early stage
Figure 1.5. SCaMCs, the most complex subgroup of MCs. In humans, four different paralogs of the ATP-Mg/Pi carrier have been described, as well as several splicing isoforms. A fifth paralog, termed SCaMC-1L, present in most mammals but absent in primates, has been recently described. In baker's yeast, a single ortholog, named Sa1lp, is found. Modified from Satrústegui et al, 2007b.

of the eukaryotic cell emergence, mediating the uptake of ATP generated by the primitive mitochondrion in exchange for phosphate, essential for the symbiont (Cavalier-Smith, 2006).

3. CALCIUM SIGNALLING

3.1. Calcium homoeostasis in the cell

Calcium is the main ion that cells use to transduce signals in the cell and plays a major role in well characterized paradigms, such as muscle contraction or neuronal synapse (Carafoli, 2002). In physiological situations, the concentration of calcium ions in the cytoplasmic compartment is kept at very low levels (50-100 nM, compared to ~1 mM in the extracellular space, Szabadkai and Duchen, 2008) by means of ATP-consuming membrane pumps (Figure 1.6) (Carafoli, 2002). Two types of calcium pumps have been described: plasma membrane calcium ATPases, that extrude calcium to the extracellular space, and sarco-endoplasmic reticulum (ER) calcium ATPases, that introduce it into the ER (Berridge et al, 2003). These pumps generate a strong calcium concentration gradient that can be used for signalling purposes. Specific extracellular or intracellular cues lead to an increase in cytoplasmic calcium levels through opening of channels in the plasma membrane and the ER. One of the main signals that stimulates calcium entry is the generation of inositol 1,4,5-triphosphate (IP3) from phosphatidylinositol 4,5-
biphosphate by activation of phospholipase C (PLC). IP3 activates IP3 receptors in the ER, which are also calcium channels, and release calcium into the cytoplasm (Berridge, 2009). In addition, calcium can also be sensed by the IP3 receptor from the lumen by the calcium-binding chaperones calreticulin and calnexin, which can regulate the IP3 receptor and the sarco-ER calcium ATPase (Berridge et al, 2003; Vangheluwe et al, 2005). Moreover, cytosolic calcium is itself the signal that activates ryanodine receptors (RyR) in the ER, further increasing cytosolic calcium levels (Bootman et al, 2002). The signal that inhibits the calcium flow from RyR is again calcium itself, since the protein detects excessive cytosolic calcium and blocks calcium gating (Clapham, 2007). In excitable cells, membrane depolarization can initially stimulate the opening of plasma membrane voltage-dependent calcium channels, which in turn can stimulate RyR (Grienberger and Konnerth, 2012). Depletion of calcium from the ER leads to activation of store operated channels from the plasma membrane to refill the ER with extracellular calcium through the recently identified STIM/Orai1 pathway (Cahalan, 2009).

3.2. Mitochondria and calcium

Initial experiments in the 60s using isolated rat mitochondria demonstrated that these organelles can...
accumulate large amounts of calcium through an energy-driven process (De Luca and Engstrom, 1961; Vasington and Murphy, 1962). It was later found that this accumulation is very limited if phosphate or ATP are absent from the assay medium, and it was proposed that calcium, phosphate and ATP form dense granules in the mitochondrial matrix (Greenawalt et al, 1964). The biological meaning of this process would be to decrease excessive cytosolic calcium levels, and mitochondria would act as “sinks” to buffer free calcium by forming inactive precipitates (Carafoli, 2010). Since the concentrations of calcium used in these experiments were much higher than those expected to be found in the cytoplasm under physiological conditions, the role of mitochondrial calcium was thought to be restricted to certain pathological situations (Carafoli, 2010). However, the finding in the 90s that cytosolic calcium signals are effectively propagated to the mitochondrial matrix revealed the importance of mitochondrial calcium in normal cell signalling (Rizzuto et al, 1993). It has since then been proposed that mitochondria can sense a 10-fold higher concentration of cytosolic calcium by the existence of microdomains between mitochondria and the ER (Rizzuto and Pozzan, 2006).

Mitochondrial calcium is now regarded as crucial in cell signalling, both in normal physiology and in several pathological situations, including amyotrophic lateral sclerosis (Kawamata and Manfredi, 2010), Alzheimer's (Contreras et al, 2010a) and Huntington's (Browne, 2008; Gandhi et al, 2009) diseases, and ischemic injury (Nakagawa et al, 2005; Schinzel et al, 2005).

### 3.2.1. Intramitochondrial calcium signalling

Calcium ions can enter the mitochondrial matrix at concentrations within the order of micromolar, in a transport through a channel that follows the kinetics of an uniporter and that can be inhibited by ruthenium red and derivatives (Gunter and Pfeiffer, 1990; Kirichok et al, 2004). Although the basic features of this process have been known and characterized for many years, the molecular components responsible are only know beginning to be identified. To date two proteins, MICU1 (mitochondrial calcium uptake 1) and MCU (mitochondrial calcium uniporter), have been demonstrated to be involved in mitochondrial calcium uptake (Baughman et al, 2011; De Stefani et al, 2011; Perocchi et al, 2010). MICU1, a protein from the inner mitochondrial membrane containing a single transmembrane and two EF-hand domains (Perocchi et al, 2010). It was identified by screening the MitoCarta database, a compendium of genes encoding proteins that are highly likely to be localized in mitochondria (Pagliarini et al, 2008), and selecting candidate genes which code for proteins located in the inner mitochondrial membrane, with ubiquitous expression and that are absent from *Saccharomyces cerevisae* (Carafoli and Lehninger, 1971) but present in vertebrates and kinetoplastids (Benaim et al, 1990; Xiong et al, 1997). A recent paper addressing the role of MICU1 indicates that it might function sensing low cytosolic calcium concentrations and preventing mitochondrial calcium uptake in resting conditions, thereby preventing calcium overload and oxidative stress (Mallilankaraman et al, 2012). As for the MCU, identified just a few months later by two independent
groups, is a two transmembrane domain protein, also located in the inner mitochondrial membrane, co-expressed with MICU1 and regarded as the channel-forming component of the calcium uptake machinery (Baughman et al, 2011; De Stefani et al, 2011).

In order to maintain calcium homeostasis, efflux mechanisms are also required in the mitochondria. Two main transport processes have been described, although, as in the case of the calcium uniporter, the identity of the genes and proteins responsible are only now beginning to be characterized: the Na⁺/Ca²⁺ exchanger and the H⁺/Ca²⁺ exchanger. The former is found particularly in excitable tissues, such as heart, brain and skeletal muscle, and catalyses the transport with an stoichiometry of 3:2 (Gunter and Pfeiffer, 1990). Its molecular identity has been recently assigned to the protein NCLX (Palty et al, 2010). On the other hand, the H⁺/Ca²⁺ exchanger is more abundant in brain, liver, lung and other non-excitatory tissues (Gunter and Pfeiffer, 1990) and the transport mechanism is electroneutral, with two protons being exchanged against one calcium ion. In this context, our laboratory partially purified two proteins of 66 and 55 kDa from rat liver mitochondria enriched in H⁺/Ca²⁺ transport activity (Villa et al, 1998). Recently, LETM1, a protein involved in the Wolf-Hirschhorn syndrome and previously characterized as an inner mitochondrial membrane K⁺/H⁺ exchanger, has been proposed as responsible for the mitochondrial H⁺/Ca²⁺ transport (Jiang et al, 2009). These authors propose that, at low mitochondrial calcium concentrations, the protein acts by introducing calcium in the matrix and extruding protons in a ruthenium red-sensitive way, while in conditions where activation of the calcium uniporter increases mitochondrial calcium, LETM1 would catalyse the efflux of calcium against the entry of protons in the matrix. However, the subject remains controversial, as most of the results in this paper diverge from previous studies on LETM1 (Hajnóczky and Csordás, 2010).

Once in the matrix, calcium promotes an overall stimulation of respiration and ATP synthesis. Concentrations of ~4 nmol calcium/mg protein can activate three dehydrogenases from the TCA cycle, namely pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase, which simulate reduced equivalents generation (McCormack et al, 1990). Calcium has also been shown to directly activate the ATP synthase in cardiomyocytes (Das and Harris, 1990).

3.2.2. Extramitochondrial calcium signalling

Recently, a novel mitochondrial regulatory mechanism through calcium, but independent of calcium entry in the mitochondria, has been identified along with the CaMCs. This group of MCs, that includes the AGCs and the SCaMCs, is characterized by the presence of EF-hand domains oriented towards the intermembrane space, where they bind cytosolic calcium (Del Arco and Satrústegui, 2004; Fiermonte et al, 2004). The sensitivity towards calcium of both types of proteins is different, with AGCs being more sensitive to calcium transients (around 100-350 nM) (Contreras et al, 2007; Pardo et al, 2006) and
SCaMCs being activated by higher calcium concentrations (around 5-30 μM) (Cavero et al, 2005; Traba et al, 2012; Traba et al, 2008; Amigo 2013), consistent with the structural differences in the EF-hand domains in both proteins (del Arco and Satrustegui, 2005; Satrustegui et al, 2007a; Contreras el al, 2007). This heterogeneous sensitivity adds complexity to the mechanism by which calcium signals can be transduced by the mitochondria: low transients promote MAS activity through activation of the AGCs, while higher concentrations are sensed by the SCaMCs and the MCU. Sustained activation of the MCU inhibits both MAS (Contreras and Satrustegui, 2009 and see below) and calcium uptake itself (Moreau and Parekh, 2008), and can finally induce permeability transition and cell death (see below).

- MAS and calcium signalling. The S0.5 for calcium activation for AGC1 and AGC2 is 280-350 nM and 100-150 nM respectively (Contreras et al, 2007). Therefore, MAS can be regulated by cytosolic calcium transients at concentrations where calcium uptake into the mitochondria does not occur, through its interaction with the EF-hand domains present in the AGC (Mármol et al, 2009; Palmieri et al, 2001; Pardo et al, 2006). The activation of MAS induces stimulation of mitochondrial respiration by transferring NADH into the matrix, and prevents the accumulation of lactate, that is synthesized from pyruvate to regenerate NAD+ and maintain glycolysis (Lasorsa et al, 2003).

At higher concentrations, calcium can enter the mitochondrial matrix through the MCU and stimulate respiration by a direct effect on three dehydrogenases of the TCA (see above). In these conditions, in tissues such as heart and brain, MAS is inhibited because the common substrate of the two pathways, α-ketoglutarate, becomes limiting and is more effectively used by the calcium-activated α-ketoglutarate dehydrogenase from the TCA than by the oxoglutarate carrier from the MAS (Contreras and Satrustegui, 2009). In liver, however, this regulatory mechanism is not observed, probably because α-ketoglutarate does not become limiting, as it can be produced by the enzyme glutamate dehydrogenase, whose activity is ~5-9-fold higher in this tissue compared to brain and heart (Contreras and Satrustegui, 2009).

- ATP-Mg/Pi carriers and calcium signalling. In normal respiring cells, the pH gradient between the mitochondrial matrix and the cytoplasm favours phosphate uptake through the phosphate carrier, while the ATP concentration is higher in the cytoplasm than in the mitochondria (Joyal and Aprille, 1992). If a calcium signal is produced under these circumstances, the ATP-Mg/Pi carrier is activated, resulting in a net influx of ATP-Mg or ADP, increasing the total amount of adenine nucleotides in the mitochondria (Aprille, 1993). However, due to the non-electrogenic nature of the transport, in situations where the pH gradient is compromised, or when the cytosolic concentrations of ATP-Mg or ADP are too low, the carrier can reverse its activity, resulting in a net efflux of adenine nucleotides from the matrix to the cytoplasm (Aprille, 1988).
The alteration of the net adenine nucleotide content within the mitochondria has been proposed to have relevant metabolic consequences. Pathways or processes with ATP-dependent steps inside the mitochondria, such as gluconeogenesis, urea cycle, synthesis of proteins, RNA and DNA, and the import of proteins, are all susceptible of being regulated by this mechanism (Aprille, 1988). State 3 respiration rates are also dependent on the mitochondrial adenine nucleotide content (Asimakis and Aprille, 1980a), and so is the calcium buffering capacity of the mitochondria (Traba et al, 2012; Zoratti and Szabó, 1995) and references therein.

3.2.3. Calcium and mitochondrial permeability transition
When mitochondria exceed their capacity for buffering calcium, a phenomenon called permeability transition (PT) occurs. PT is characterized by loss of mitochondrial membrane potential, swelling, efflux of solutes under 1500 Da, release of pro-apoptotic factors and, eventually, cell death (Halestrap, 2009). Although calcium is the main inducer of PT, several regulatory elements have been described, including pH, divalent cations, reactive oxygen species and adenine nucleotides (Rasola and Bernardi, 2011). Its occurrence has been involved in many paradigms where calcium overload-induced cell death plays an important role, such as stroke (Schinzel et al, 2005), Parkinson's disease (Gandhi et al, 2009) or glutamate excitotoxicity (Li et al, 2009). The process of PT is mediated by the formation/activation of a non-specific channel in the mitochondria termed permeability transition pore (PTP), whose components are still mainly unknown (Baines, 2009b). Among the proteins postulated to be important in its structure, both the ANT and the voltage-dependent anion channel have been shown to be dispensable for occurrence of PT (Baines et al, 2007; Kokoszka et al, 2004). As for the regulatory components, the most significant protein unambiguously involved in the modulation of PTP opening is cyclophylin D (CypD), a peptidyl prolyl-isomerase from the mitochondrial matrix whose lack renders mitochondria more resistant to PT (Baines et al, 2005; Basso et al, 2005; Nakagawa et al, 2005; Schinzel et al, 2005). The means by which CypD exert its modulation on PT are not well known. It has been suggested that CypD ablation or its blockade by its pharmacological inhibitor, cyclosporin A, unmasks an an inhibitory site for phosphate, which otherwise acts as a PTP inducer (Basso et al, 2008), a site that has been proposed to be located in the ATP synthase (Chinopoulos and Adam-Vizi, 2012). However, other laboratories have failed to reproduce the experiments supporting these results (McGee and Baines, 2012; Varanyuwatana and Halestrap, 2012) and the mechanism of action of CypD remains unknown.

4. PHYSIOLOGY AND PATHOLOGY OF THE MITOCHONDRIAL ATP-Mg/Pi CARRIERS
By changing the net adenine nucleotide content from the mitochondria, the ATP-Mg/Pi carrier could, in theory, regulate all matrix reactions that require ADP and ATP (Aprille, 1988). Experiments in the 80s and 90s using isolated mitochondria have prompted our understanding of the features of the transport reaction catalysed by the carrier and the correlation between matrix adenine nucleotide content and different
adenine nucleotide-dependent reactions, such as pyruvate carboxylation (Aprille et al, 1981), citrulline synthesis (Goldstein and Aprille, 1982) or intramitochondrial protein synthesis (Joyal et al, 1995). In recent years, identification of the SCaMC genes and proteins is allowing us to describe more accurately the known functional roles of the different paralogs and to unveil new unexpected functions.

4.1. Sal1p: the mitochondrial ATP-Mg/Pi carrier in S. cerevisae
Yeast posses a single form of the ATP-Mg/Pi carrier, termed Sal1p which, like its mammalian counterparts, is located in the inner mitochondrial membrane, has a structure with six transmembrane domains and posses EF-hand motifs on its N-terminal (Cavero et al, 2005; Chen, 2004). Sal1p has been shown to mediate the exchange of ATP-Mg with inorganic phosphate in yeast mitochondria, but with higher calcium requirements than mammalian SCaMCs (S0.5 of ~30 μM (Cavero et al, 2005), compared to 5-15 μM found in SCaMC-1 and SCaMC-3 (Traba et al, 2012; Amigo et al, 2013), probably due to the presence of only two canonical EF-hand domains, whereas mammalian paralogs have four (Cavero et al, 2005). Although Sal1p activity is dispensable, it becomes essential in the absence of Aac2p, the main form of the ATP/ADP carrier in yeast, and calcium-binding to Sal1p has been shown to be required for its function (Chen, 2004). It has been proposed that lethality in these conditions is due to absence of a mechanism for ATP import into the mitochondria, where it is required for protein synthesis (Cavero et al, 2005; Traba et al, 2009a; Traba et al, 2011), Recently, Kucejova and collaborators have pointed to loss of mitochondrial DNA as the cause for lethality in strains defective in Aac2p and Sal1p, although the authors discard a role for ATP import, as they did not observe any differences in the levels of mitochondrial adenine nucleotides in these conditions (Kucejova et al, 2008).

Sal1p is also a target of glucose-induced calcium signals in yeast. Glucose binding to G protein-coupled receptor Gpr1p induces a cytosolic calcium signal which is mediated by activation of PLC, release of IP3 and calcium influx from the extracellular space (Ansari et al, 1999; Lemaire et al, 2004; Tisi et al, 2002; Tisi et al, 2004). Cavero and collaborators have shown that when all the paralogs of the ATP/ADP carrier are absent (i.e. in conditions where Sal1p is indispensible) the stimulating effect of glucose in bud formation is completely abolished by pre-treatment of the cells with EGTA, an effect that is not observed in wild type cells or in strains deficient in Sal1p (Cavero et al, 2005; Granot and Snyder, 1993). This is the only known target of the glucose-induced calcium signal in yeast identified to date.

4.2. SCaMC-2 and thermogenesis
A mouse model deficient in SCaMC-2 has been recently described by Anunciado-Koza and collaborators. Mice lacking SCaMC-2 are viable, fertile and with no apparent phenotype (Anunciado-Koza et al, 2011). These authors previously observed that SCaMC-2 is overexpressed in inguinal fat and skeletal muscle from mice deficient in UCP1 and leptin, and hypothesized that the protein could be important for
thermogenesis (Anunciado-Koza et al, 2011). Their results using SCaMC-2\(^{-/-}\) mice show that its absence does not alter glucose tolerance, insulin resistance or normal body temperature maintenance, but animals become resistant to diet-induced obesity and have reduced physical endurance on a treadmill. Moreover, mouse embryonic fibroblasts derived from SCaMC-2\(^{-/-}\) mice show reduced calcium efflux from the ER after treatment with bradykinin, but not other ER mobilizing-agents, such as thapsigargin or ionomicyn, and mild glycolytic metabolism (Anunciado-Koza et al, 2011). Co-expression of SCaMC-1 and SCaMC-2 in skeletal muscle, the main tissue where SCaMC-2 is detected (Del Arco and Satrústegui, 2004), could underlie the absence of more severe defects. A compensation effect by UCP2 in UCP1\(^{-/-}\) mice is also believed to be responsible for the mild phenotype observed, as mice are not hyperphagic nor prone to obesity as expected (Enerbäck et al, 1997).

In the same line of evidence, SCaMC-2 has been suggested to be involved in winter adaptation of the freeze-resistant rainbow smelt, as its expression is enhanced in liver in response to cold temperatures (Richards et al, 2008).

4.3. SCaMC-1 and mitochondrial calcium retention

Calcium is accumulated in the mitochondrial matrix by the formation of calcium-phosphate precipitates, a process which requires ATP (Carafoli et al, 1965 and see above). Therefore, by changing the mitochondrial adenine nucleotide content, the ATP-Mg/Pi carrier can regulate the susceptibility of mitochondria to undergo PT. Some years ago, Hagen and collaborators proposed that part of the effect of phosphate as a PT inducer could be mediated by adenine nucleotide efflux through the ATP-Mg/Pi carrier activated by the presence of calcium (Hagen et al, 2003). Moreover, up-regulation of SCaMC-1 has been observed in non-metastatic and metastatic breast cancers (Chen et al, 2011), arguing for a possible role of the protein in the observed resistance to cell death in transformed cells (Hanahan and Weinberg, 2011). Direct evidence for this has been recently obtained by Traba and collaborators, who have shown that high levels of SCaMC-1 positively correlate with resistance to calcium-induced cell death, whereas knock-down of the transporter reduces mitochondrial calcium retention capacity and promotes cell death (Traba et al, 2012).

4.4. The mitochondrial ATP-Mg/Pi carrier and liver metabolism

Rat liver is, by far, the main tissue where the ATP-Mg/Pi carrier has been studied. It is also the tissue where its activity was first identified, where it was described to play an important role is in the filling of adenine nucleotides that takes place in mammalian liver mitochondria during the first post-natal hours (Aprille and Asimakis, 1980; Sutton and Pollak, 1978; Sutton and Pollak, 1980). Two complementary signals have been proposed to mediate the process. First, the increase in cytosolic ATP levels and the ATP/ADP ratio resulting from the release of hormones that promote glycogenolysis and glycolysis, such as glucagon and adrenaline, which are secreted shortly after birth (Cuezva et al, 1982; Sutton and Pollak,
Second, inflation of the lungs at birth, which provokes a sudden perfusion of the liver with well oxygenated blood and allows electron transport chain activity and the generation of a mitochondrial proton gradient (Aprille, 1990). The formation of the proton gradient allows entry of phosphate into the mitochondria through the phosphate carrier, which can be then exchanged with cytosolic ATP through the ATP-Mg/Pi carrier activated by hormone-induced calcium signals (Aprille, 1993).

Consistently, the ATP-Mg/Pi carrier has been proposed to be a target of glucagon and calcium-mobilizing agents, as it has been observed that net adenine nucleotide content in liver mitochondria increases in response to these signals (Aprille et al, 1987; Bryla et al, 1977; Titheradge and Haynes, 1980; Titheradge et al, 1979), and that adenine nucleotide uptake is abolished by calcium chelating agents (Haynes et al, 1986). However, a mechanism independent of the ATP-Mg/Pi carrier has been proposed, according to which, calcium entry in the mitochondria inhibits mitochondrial pyrophosphatase, leading to an accumulation of intramitochondrial pyrophosphate that is exchanged by cytosolic adenine nucleotides through the ANT (Davidson and Halestrap, 1988).

In addition, Aprille and collaborators have described correlations between mitochondrial adenine nucleotide content and adenine nucleotide-dependent metabolic activities in the mitochondrial matrix, such as pyruvate carboxylation (Aprille et al, 1981) and citrulline synthesis (Goldstein and Aprille, 1982), which are part of the gluconeogenic and ureogenic pathways, respectively.
INTRODUCCIÓN
1. LA MITOCONDRIA

La mitocondria es un órgano de doble membrana presente en las células eucariotas, donde desarrolla un papel importante en la generación de energía en forma de ATP, así como en la regulación de procesos como la apoptosis, la homeostasis de calcio y la proliferación celular. La mitocondria genera también intermediarios metabólicos que intervienen en diferentes vías, tales como el metabolismo de aminoácidos y la síntesis de centros de hierro-azufre.

Actualmente está ampliamente aceptado que la mitocondria evolucionó a partir de la bacteria aeróbica a-proteobacteria mediante un proceso de endosimbiosis (Sagan, 1967), y su origen se encuentra estrechamente unido a la aparición de la célula eucariota, que tuvo lugar hace 1.6-2 billones de años, en la transición de una atmósfera reductiva a una oxidante (Knoll et al, 2006). Se cree que la toxicidad del oxígeno impulsó la asociación de un huesped aneróbico y un endosimbionte aeróbico que evolucionó hacia la actual mitocondria, reduciendo de esta manera la tensión local de oxígeno (Andersson et al, 2003). Una de las pruebas más importantes del origen endosimbiótioco de las mitocondrias es la existencia de material genético en su interior, aunque reducido, con un número de genes que oscila entre los 2 de Plasmodium falciparum hasta los 67 de Reclinomonas americana, la mayoría de los cuáles codifican para proteínas relacionadas con la respiración aerobia y la traducción mitocondrial (Gray et al, 1999; Andersson et al, 2003). Se han identificado organismos eucariotas sin mitocondrias, aunque en todos los casos de ha demostrado que su ausencia es consecuencia de la pérdida o reducción del orgánulo (Van der Giezen et al, 2002; Van der Giezen y Tovar, 2005; Hrdy et al, 2004). Dos orgánulos derivados de la mitocondria se han descrito hasta el momento: los mitosomas y los hidrogenosomas. Ambos han perdido el material genético y la mayoría de las enzimas de relacionadas con la fosforilación oxidativa (Dyall y Johnson, 2000; Boxma et al, 2005). Los hidrogenosomas se han detectado en el ciliado Nyctotherus ovalis, el hongo Neocallimastix frontalis, el flagelado Psalterionomas lanterna y los patógenos humanos Trichomonas vaginalis y Tritrichomonas foetus, e intervienen en la síntesis anerobia de ATP por fosforilación a nivel de sustrato usando hidrógeno como acceptor final de electrones (Shiflett y Johnson, 2010). Recientemente se han identificado orgánulos similares a hidrogenosomas en tres nuevas especies del filum Loricifera que constituyen los únicos metazoos anaerobios descritos hasta la fecha (Danovaro et al, 2010). Por otro lado, los mitosomas se encuentran en el flagelado Giardia lamblia, el parásito intestinal Entamoeba histolytica y en algunas especies de Microsporidia, y no parecen desempeñar ningún papel en la síntesis de ATP, sino únicamente en la generación de centros de hierro-azufre (Tovar et al, 2003), una función celular indispensable que se ha propuesto como la característica común de todos los orgánulos derivados de la mitocondria (Shiflett y Johnson, 2010; Lill y Kispal, 2000). De acuerdo con su menor complejidad funcional, el número de proteínas de los mitosomas es más reducido que el de las mitocondrias o los hidrogenosomas (Jedelský et al, 2011; Schneider et al, 2011).
Además del menor número de genes, una diferencia fundamental entre la a-proteobacteria y la mitocondria es la presencia de proteínas de transporte en la membrana de estas últimas, lo que le permite intercambiar solutos con el citosol, y cuya inserción debió ser un paso importante en el proceso de endosimbiosis (Cavalier-Smith, 2006).

2. TRANSPORTADORES MITOCONDRIALES

La membrana mitoconrial interna es relativamente impermeable y requiere la presencia de proteínas específicas para catalizar el intercambio de solutos entre el citoplasma y la matriz mitoconrial (Kunji, 2004; Palmieri, 2004; Del Arco and Satrústegui, 2005) (Figura 1.1). Estas proteínas, denominadas transportadores mitocondriales (MCs) y agrupadas en la familia SLC25, se originaron por duplicaciones en tandem a partir de un antecesor común, y se caracterizan por la presencia de tres repeticiones de unos 100 aminoácidos, cada uno de los cuales incluye dos dominios transmembrana unidos por un largo loop hidrofílico, y por la presencia de una secuencia conservada característica (PROSITE: PS50920): Px(D/E)xh(K/R)x(R/K)x20–30(D/E)Gx4a(K/R)GRG, donde “X” es un aminoácido cualquiera, “h” un aminoácido hidrofílico y “a” un aminoácido aromático (Indiveri et al, 1997).

En el genoma de la levadura se han identificado 34 posibles MCs con 27 actividades transportadoras

![Figura 1.1. La familia de transportadores mitocondriales. Representación esquemática de la membrana mitoconrial interna con todos los transportadores mitocondriales y algunas de las rutas metabólicas en las que intervienen. Modificado de Palmieri, 2008.](image-url)
diferentes, mientras que en humanos se han encontrado 50 miembros, varios de los cuáles no tienen todavía sustratos conocidos (Del Arco y Satrústegui, 2005; Palmieri et al, 2006). Hasta hace poco tiempo se pensaba que los MCs se encontraban exclusivamente en eucariotas, aunque recientemente se ha descrito un MC que cataliza el transporte unidireccional de ATP en Legionella pneumophila, donde probablemente apareció por transferencia horizontal (Dolezal et al, 2012). Según el sustrato que transportan, los MCs se subdividen en cuatro familias, que también comparten similitud a nivel de secuencia: 1) transportadores de nucleótidos y dinucleótidos 2) transportadores de cetoácidos, 3) transportadores de aminoácidos y 4) transportadores de otros sustratos (Robinson y Kunji, 2006). La primera de estas subfamilias, y en particular el grupo de transportadores de nucleótidos de adenina, constituye el subgrupo más complejo de MCs (Del Arco y Satrústegui, 2005; Traba et al, 2011; Satrústegui et al, 2007b).

El defecto en el transporte de algunos MCs se encuentra asociado a condiciones patológicas, como en los casos de la microcefalia amish, causada por mutaciones en el gen SLC25A19, que codifica para el carrier de tiamina pirofosfato, el síndrome HHH, producido por mutaciones en el gen SLC25A19, que codifica para el carrier de orinitina/citrulina, o la epilepsia mioclónica neonatal, producida por fallos en el gen SLC25A22 del carrier de glutamato (Palmieri, 2008).

2.1. Estructura y mecanismo de transporte
La mayoría de los MCs catalizan un antiporte 1:1, aunque algunos transportadores pueden también mediar un transporte unidireccional (i.e. transportador de carnintina/acil carnitina y algunas formas del transportador de ATP/ADP) y algunos catalizan el simporte con H⁺ (transportadores de fosfato, glutamato y piruvato) (Palmieri, 2004). Funcionalmente, hasta hace poco se pensaba que los transportadores actuaban como homodímeros, lo que explicaría cómo proteínas relativamente pequeñas, de sólo seis hélices transmembrana, pueden catalizar el transporte de sustratos grandes (Kotaria et al, 1999; Schroers et al, 1998). Sin embargo, la estructura cristalográfica del transportador de ATP/ADP (ANT) unido a su inhibidor carboxiatractilósido indica que la proteína forma una estructura circular con una pseudosimetría ternaria a partir de las triples repeticiones, y además carece de dominios de dimerización (Pebay-Peyroula et al, 2003). A partir del análisis de esta estructura, Kunji y colaboradores han propuesto un modelo según el cuál los transportadores funcionarían como monómeros (Kunji y Crichton, 2010). En concordancia con el modelo, sus resultados indican que el transportador de ATP/ADP de levaduras funciona como monómero en detergentes suave (Bamber et al, 2007), y la estructura en dos dimensiones por microscopía electrónica del transporte de ATP/ADP de levaduras unido a su inhibidor atractilósido también parece indicar una posible conformación monomérica (Kunji y Harding, 2003).

La similitud en las secuencias de los miembros de la familia de MCs sugiere que comprueban el mismo
mecanismo de transporte. Según el modelo de Kunji, dos grupos de residuos presentes en todos los transportadores son importantes: uno es la secuencia PX[DE]XX[RK], localizada en las hélices impares, en el lado de la proteína orientado hacia la matriz mitocondrial, mientras que el otro es la secuencia [FY][DE]XX[RK], situada en las hélices pares, hacia el lado del espacio intermembrana. Estas secuencias forman puentes salinos entre ellas, y es la formación y ruptura de estos enlaces la que se cree que media el transporte de los sustratos (Figura 1.2) (Robinson et al, 2008).

Figura 1.2. Mecanismo de transporte de los transportadores mitocondriales (MC). A. Representación de las hélices de la estructura de un MC. Las hélices H1-H6 corresponden a los dominios transmembrana, mientras que las hélices H12, H34 y H56 son hélices más cortas que se encuentran en las secuencias que unen los dominios transmembrana en el lado de la matriz. Los números indican los residuos donde comienzan y terminan las hélices grandes en SCA-MC-3, un miembro de la familia MC. B. Representación de la simetría ternaria en el transportador de ATP/ADP vista desde el espacio intermembrana y la membrana mitocondrial. Las hélices impares, pares y de la matriz están coloreadas en rojo, azul y verde respectivamente. La red de puentes salinos del fondo de la cavidad se indica con la letra S. La representación dentro del cuadrado indica los residuos cargados positiva y negativamente del motivo característico de los MC, que es el que forma los puentes salinos (línea roja punteada) al fondo de la cavidad. Una molécula de ADP se encuentra unida al sitio de unión de sustrato propuesto en los puntos de contacto I, II y III. C. Cadenas laterales del transportador de ATP-Mg/Pi y su sustrato principal, ATP-Mg, representado como barras y el Mg^{2+} como una esfera. La coordinación del ión de magnesio requiere la intervención de una
2.2. Transportadores mitocondriales regulados por calcio

Un subgrupo de MCs está caracterizado por poseer dominios de unión a calcio de tipo manos EF en su N-terminal, orientados hacia el espacio intermembrana, lo que les hace susceptibles de ser regulados por calcio citosólico (Figura 1.3) (revisado en Satrústegui et al, 2007b). Estos transportadores mitocondriales regulados por calcio (CaMCs) son los transportadores de aspartato/glutamato (AGCs) y los transportadores de ATP-Mg/Pi, denominados SCaMCs de su acrónimo en inglés short calcium-binding mitochondrial carriers. Los AGCs poseen cuatro pares de dominios de manos EF, además de un dominio vestigial (Contreras et al, 2007), mientras que los SCaMCs posee sólo cuatro dominios de manos EF, que además muestran gran similitud con los de calmodulina (Del Arco y Satrústegui, 2004). El transporte catalizado por ambos tipos de transportadores fue descrito hace varias décadas, pero la identificación molecular de las proteínas sólo ha tenido lugar recientemente (Del Arco y Satrústegui, 1998; Del Arco et al, 2000; Del Arco y Satrústegui, 2004; Fiermonte et al, 2004). En 2011 se identificó un nuevo miembro del grupo de CaMCs en la membrana del cloroplasto que posee un único dominio de manos EF y se cree que cataliza el importe de S-adenosil-metionina al interior del cloroplasto (Bayer et al, 2011; Stael et al, 2011).

2.2.1. Los transportadores de aspartato/glutamato. Los AGCs catalizan el intercambio electrogénico de una molécula de aspartato por una molécula de glutamato más un protón. Este transporte es irreversible y constituye el paso regulador de la lanzadera de aspartato/malato (MAS), que introduce NADH de origen citosólico en la mitocondria. Las bases de este transporte fueron sentadas hace cuarenta años por el grupo de Katherine LaNoue (LaNoue y Williamson, 1971; LaNoue y Tischler, 1974).

Figura 1.3. Transportadores mitocondriales regulados por calcio (CaMCs). Los CaMCs se caracterizan por la presencia de dominios de tipo manos EF en su extremo N-terminal, además de la estructura típica de los transportadores mitocondriales, con seis dominios transmembrana en su extremo C-terminal y ambos extremos orientados hacia el lado del espacio intermembrana. Hay dos tipos de CaMCs: los transportadores de aspartato/glutamato, o AGCs, y los transportadores de ATP-Mg/Pi, denominados SCaMCs (acrónimo en inglés de short calcium-binding mitochondrial carriers). Tomada de Satrústegui et al, 2007b.
En mamíferos hay dos parálisis del AGC, cada uno de los cuáles tiene un patrón de expresión diferente en adultos: aralar o AGC1 (SLC25A12), es la forma más abundante en tejidos excitable, en concreto en cerebro y en músculo esquelético (Del Arco y Satrústegui, 1998; Palmieri et al, 2001), mientras que citrina, o AGC2 (SLC25A13) se expresa fundamentalmente en hígado y riñón (Del Arco et al, 2000). Ambas proteínas se co-expresan en corazón (Del Arco y Satrústegui, 1998; Del Arco et al, 2000). En levaduras sólo existe una forma, denominada Agc1p, que carece de dominios de manos EF y que cataliza, además del intercambio aspartato/glutamato, el transporte unidireccional de glutamato y, en menor medida, el de aspartato (Cavero et al, 2003). En humanos, mutaciones en el gen de citrina se encuentran asociados con la citrulinemia de desarrollo adulto de tipo II (CTLN2) (Kobayashi et al, 1999), mientras que la deficiencia en aralar es muy poco común y hasta la fecha sólo se ha identificado un paciente con ella (Wibom et al, 2009).

Las mitocondrias procedentes de músculo esquelético y cerebro de ratones deficientes en aralar muestran una reducción en la respiración usando glutamato y malato como sustratos, y no tienen actividad detectable de MAS (Jalil et al, 2005). Además, aralar se requiere para la correcta síntesis de mielina en el cerebro (Satrústegui et al, 2007a, Satrústegui et al, 2007b) y los ratones deficientes en aralar muestran defectos en mielinización que se manifiestan poco después del nacimiento, con niveles de aspartato y N-acetil aspartato (NAA), que son precursores de la síntesis de mielina, drásticamente reducidos (Jalil et al, 2005; Sakurai et al, 2010). Para explicar estas observaciones, se ha propuesto un modelo según el cuál la mitocondria de las neuronas produce aspartato, que es transportado hacia el citoplasma mediante aralar y convertido a NAA por la enzima aspartato-N-acetil transferasa. A continuación, el NAA es transportado transaxonalmente a los oligodendrocitos, donde proporciona grupos acetil para la síntesis de los lípidos de la mielina (Satrústegui et al, 2007a). La razón por la cuál los fallos en la síntesis de mielina en el ratón deficiente en aralar sólo tienen lugar después del nacimiento es probablemente el solapamiento en la expresión de citrina y aralar durante durante el desarrollo prenatal (Del Arco et al, 2002), además de la existencia de una barrera hematoencefálica más permeable, que sólo se consolida después del nacimiento (Ramos et al, 2003). A partir de ese momento, el cerebro pasa a ser estrictamente dependiente de la producción endógena de aspartato. En el único paciente con defectos en aralar descrito hasta la fecha también se observan fallos en mielinización, además de una severa hipotonía muscular y respiración mitocondrial disminuida con glutamato y malato como sustratos (Wibom et al, 2009).

Además, la deficiencia en la actividad de aralar podría subyacer a otras dos condiciones patológicas: el síndrome de Mohr-Tranejaer y el autismo. El síndrome de Mohr-Tranejaer se caracteriza por un importe deficiente de proteínas en la membrana mitocondrial interna a través del complejo DDP1/TIMM8a-TIM13 (Koehler et al, 1999; Roesch et al, 2002), y se ha demostrado que los AGCs se
importan a través de esta vía (Roesch et al, 2004). En cuanto al autismo, se han descrito dos SNPs en el gen de aralar asociados a autismo (Ramoz et al, 2004; Segurado et al, 2005; Silverman et al, 2008; Turunen et al, 2008), aunque otros grupos no han encontrado este vínculo (Blasi et al, 2006; Correia et al, 2006; Rabionet et al, 2006) (revisado en Rossignol y Frye, 2012). Un estudio reciente con tejidos post-mortem de pacientes humanos también ha relacionado aralar con autismo, aunque en este caso los autores indican que el incremento en la actividad de AGC observado en pacientes autistas es debido a la presencia de concentraciones anormalmente altas de calcio en el neocórtex, y no a mutaciones o polimorfismos en el gen de aralar (Palmieri et al, 2010).

Por otro lado, mutaciones en AGC2 producen CTLN2, una enfermedad hepática de desarrollo en adultos encontrada fundamentalmente entre la población japonesa (Kobayashi et al, 1999). Está causada por defectos en la actividad de la enzima arginosuccinato sintasa y clínicamente caracterizada por hiperamonemia y niveles elevados de citrulina en el suero (Kobayashi et al, 1999). La deficiencia en citrulina afecta a la síntesis de urea, al reducir el flujo de aspartato desde la mitochondria al citoplasma, donde se requiere para la formación de arginosuccinato a partir de citrulina (Palmieri et al, 2001). Además, como en el caso de aralar, la falta de citrulina produce un importe deficiente de NADH citósólico a la mitochondria, lo que podría estimular la actividad de otras lanzaderas de NADH, concretamente las lanzadera de glicerol-fosfato y de citrato/malato (Saheki y Kobayashi, 2002). En este último caso, la lanzadera de citrato/malato consume NADH citósólico, pero produce acetil-CoA en el citoplasma, donde se emplea en la síntesis de ácidos grasos, lo que explicaría la hiperlipidemia y el hígado graso observado en muchos pacientes de CTLN2 (Saheki et al, 2004).

Una enfermedad diferente, denominada coleostasis intrahepática neonatal se ha observado en neonatos con la misma mutación en citrulina que la de pacientes CTLN2 (Ohura et al, 2001; Tomomasa et al, 2001). Los ratones deficientes en citrulina tienen un fenotipo de baja actividad de MAS, con niveles reducidos de gluconeogenesís a partir de lactato, pero no de piruvato, y una disminución en la producción de urea, pero no presentan las características patológicas de la CTLN2 (Sinasac et al, 2004).

2.2.2. Los transportadores de ATP-Mg/Pi. El transportador mitocondrial de ATP-Mg/Pi cataliza el intercambio de fosfato inorgánico (HPO₄²⁻) con ATP-Mg² o HADP². Esta actividad es insensible a los inhibidores de ANT carboxiáctractilósido y ácido bonkreikiko (Austin y Aprille, 1984), es estrictamente dependiente de la presencia de concentraciones de calcio del orden micromolar (Nosek et al, 1990) y tiene lugar de forma no electrogénica, siendo por lo tanto reversible y dependiente de los gradientes de concentración de los sustratos (Joyal y Aprille, 1992) (Figura 1.4A). Este transporte fue inicialmente descrito por Sutton y Pollack a finales de los años 70 (Sutton y Pollak, 1978) y caracterizado en detalle por
el grupo de June Aprille durante las dos décadas siguientes (Asimakis y Aprille, 1980b; Nosek y Aprille, 1990; Joyal y Aprille, 1992; Dransfield y Aprille, 1994; Austin y Aprille, 1994; Joyal et al, 1995). Cuando se activa, el transportador altera el contenido neto de nucleótidos de adenina de la mitocondria, regulando de esta manera reacciones mitocondriales y vías con pasos dependientes de ATP (Aprille, 1988) (Figura 1.4B). Aunque su sustrato principal es ATP unido a magnesio, el transportador también puede transportar ADP y, en menor medida, AMP (Asimakis y Aprille, 1980a). Debido a su relativa menor actividad comparada con la ATP sintasa y con el ANT, Aprille y colaboradores afirmaron que el transportador no afecta al ratio ATP/ADP de los diferentes compartimentos, sino que únicamente afecta a los niveles totales de nucleótidos de adenina (Aprille, 1993), aunque Schild y colaboradores observaron una correlación positiva entre el ratio ATP/ADP y el contenido total de nucleótidos de adenina a velocidades intermedias

**Figura 1.4. El transportador de ATP-Mg/Pi. A.** Representación esquemática de la actividad del transportador de ATP-Mg/Pi. El transporte catalizado por el transportador altera el contenido neto de nucleótidos de adenina. La dirección del transporte está regulada por la concentración de sustratos, ATP-Mg (o ADP) y fosfato. Dado que el transporte de fosfato a través del transportador de fosfato es un proceso rápido y dependiente del gradiente de protones entre la matriz y el citosol, la actividad del transportador se encuentra indirectamente regulada por el estado energético de la mitocondria. Los nucleótidos de adenina transportados no alteran el ratio ATP/ADP, afectando únicamente al contenido neto de nucleótidos de adenina. **B.** Representación de reacciones dependientes de nucleótidos de adenina que tienen lugar en la matriz mitocondrial, entre las que se incluyen los primeros pasos de gluconeogenesis y ureogenesis, el importe de proteínas, la síntesis de proteínas intramitocondriales y la fosforilación oxidativa.
Figura 1.5. SCaMCs, el subgrupo más complejo de transportadores mitocondriales. En humanos, cuatro parálogos diferentes han sido descritos, así como varias formas de splicing. Un quinto parálogo, denominado SCaMC-1L, presenta en la mayoría de mamíferos, pero ausente en primates, ha sido recientemente descrito. En levadura existe un único ortólogo, denominado Sal1p. Modificado de Satrústegui et al, 2007b.

de respiración (Schild et al, 1999).

El primer paso hacia la caracterización molecular del transportador de ATP-Mg/Pi tuvo lugar con la identificación de un transportador que une calcio denominado Effinal, descrito por microscopía electrónica como presenta en la membrana de peroxisomas en el intestino delgado de conejo (Weber et al, 1997). Unos años después, los genes y proteínas responsables por la actividad mitocondrial transportadora de ATP-Mg/Pi en humanos se identificaron como los pequeños transportadores mitocondriales dependientes de calcio o SCaMCs (Del Arco y Satrústegui, 2004; Fiermonte et al, 2004; Traba et al, 2009b; Haitina et al, 2006; Amigo et al, 2012). Este grupo de proteínas presenta gran complejidad en humanos e incluye cinco parálogos y múltiples formas de splicing (Figure 1.5) (Satrústegui et al, 2007b): SCaMC-1 (SLC25A24, también denominada APC1) y SCaMC-2 (SLC25A25, también denominada APC3), que presentan diferentes isoformas de splicing por el uso de promotores alternativos (Del Arco y Satrústegui, 2004); SCaMC-3 (SLC25A23, también denominada APC2), con múltiples formas de splicing que se diferencian en su C-terminal (Del Arco, 2005); SCaMC-3L (SLC25A41), que carece de dominios de manos EF y no está regulada por calcio (Traba et al, 2009b); y SCaMC-1L, el miembro más
recentemente identificado, que aparece en mamíferos no primates y que en humanos se encuentra en forma de pseudogen, y que en roedores presenta una expresión específica en testículos, donde tiene varias localizaciones subcelulares durante el desarrollo de la espermátida (Amigo et al, 2012). En levaduras tan sólo hay un único parálogo, denominado S1lp.

Se ha indicado que el transportador de ATP-Mg/Pi podría haber jugado un papel importante en la aparición de la célula eucariótica, mediando el transporte de ATP generado en el hospedador hacia la primitiva mitocondria en intercambio por fosfato (Cavalier-Smith, 2006).

3. SEÑALIZACIÓN POR CALCIO

Figura 1.6. El calcio en la célula. El calcio se mantiene a niveles muy bajos en el citoplasma mediante el empleo de ATPasas de calcio de la membrana plasmática (PCMA) y del retículo endoplasmático (SERCA). La concentración de calcio puede incrementarse por la entrada a través de canales activados por voltaje, en el caso de tejidos excitables, o por canales estimulados por receptor. Además, el calcio citósólico puede aumentar por la activación de canales inducidos por agonistas que estimulan la vía PLC-IP3. IP3 puede actuar sobre el receptor de IP3 del retículo endoplasmático (IP3R) e inducir la liberación de calcio del retículo endoplasmático, que a su vez puede activar un mayor flujo a mediante la activación de los receptores de ryanodina (RyR). El llenado de calcio del retículo endoplasmático tiene lugar a través de la vía STIM-Orai1. Los aumentos de calcio citósólico también favorecen la captación de calcio por la mitocondria a través del uniportador de calcio (MCU). La homeostasis de calcio en la matriz se consigue por dos mecanismos de eflujo: el intercambiador H⁺/Ca²⁺ y el Na⁺/Ca²⁺. Si el calcio excede la capacidad de retención de la mitocondria, tiene lugar la apertura del poro de permeabilidad de transición (PTP). Imagen tomada de Orrenius et al, 2003.

3.1. Homestasis de calcio en la célula

El calcio es el principal ion utilizado por la célula para transducir señales y juega un papel importante en paradigmas como la contracción muscular o la sinapsis neuronal (Carafoli, 2002). En situaciones fisiológicas, la concentración de iones de calcio en el citoplasma se mantiene a concentraciones muy bajas (50-100 nM, frente a 1 mM del espacio extracelular (Szabadkai y Duchen, 2008) mediante bombas de membrana que consumen ATP (Carafoli, 2002) (Figura 1.6). Dos tipos de bombas de calcio se han descrito: las ATPasas de la membrana plasmática, que expulsan calcio al espacio extracelular, y las
ATPasa sarco-reticulares, que lo introducen al ER (Berridge et al, 2003). Estas bombas generan un fuerte gradiente de concentración que puede ser empleado para producir señales. Estímulos extracelulares o intracelulares específicos conducen a un incremento del calcio citósólico mediante la apertura de canales de la membrana plasmática y el ER. Una de las señales estimuladoras para la entrada de calcio es la producción de inositol 1,4,5-trifosfato a partir de fosfatidil inositol 4,5-bifosfato mediante la activación de fosfolipasa C (PLC). El IP3 activa sus receptores en la membrana del ER, que a su vez son canales de calcio y liberan calcio al citosol (Berridge, 2009). Además, el calcio del lumen del ER es reconocido mediante chaperonas que unen calcio, como calreticulina y calnexina, lo que permite a estas proteínas regular desde el interior del orgánulo al receptor de IP3 y a la ATPasa sarco-ER (Berridge et al, 20003; Vangheluwe et al, 2005). Además, el aumento de calcio citósólico produce la activación de los receptores de rianodina (RyR) en el ER, lo que conduce a un aumento todavía mayor de los niveles de calcio (Bootman et al, 2002). Cuando los niveles de calcio son excesivos, los RyR se inhiben (Clapham, 2007).

En células excitable, la despolarización de la membrana plasmática estimula la apertura de canales de calcio dependientes de voltaje, que a su vez estimulan los RyR. La depleción de los niveles de calcio del ER induce la activación de los canales de calcio operados por almacenamiento de la membrana plasmática para rellenar el ER con calcio extracelular a través de la recién identificada vía STIM/Orai1 (Cahalan, 2009).

3.2. Mitocondrias y calcio

Durante los años 60, experimentos usando mitocondrias de rata demostraron que estos orgánulos podían acumular largas cantidades de calcio a través de un proceso dependiente de energía (De Luca y Engstron, 1961; Vasington y Murphy, 1962). Se halló más tarde que esta acumulación era muy limitada si no había fosfato y ATP en el medio de ensayo, y se propuso que el calcio, el fosfato y el ATP forman gránulos densos en la matriz mitocondrial (Greenawall et al, 1964). El significado biológico de este proceso sería la disminución de niveles de calcio citósólicos excesivos, que son perjudiciales para la célula. De esta manera, las mitocondrias funcionarían como “sumideros” para tamponar el nivel de calcio libre mediante la formación de precipitados inactivos (Carafoli, 2010). Dado que la concentración de calcio requerida en estos experimentos era mucho mayor que la que se espera encontrar en el citoplasma en condiciones fisiológicas, se pensó que el papel del calcio mitocondrial estaba restringido a determinadas condiciones patológicas (Carafoli, 2010). Sin embargo, el hallazgo en los años 90 de que las señales normales de calcio citósólicas se propagan a la matriz mitocondrial reveló la importancia del calcio mitocondrial en la señalización celular en condiciones fisiológicas (Rizzuto et al, 1993). Desde entonces se ha propuesto que la mitocondria puede detectar una concentración 10 veces mayor que la citósólica debido a la presencia de microdominios entre el ER y la mitocondria (Rizzuto y Pozzan, 2006).

Hoy en día el calcio mitocondrial se considera fundamental en la señalización celular, tanto en condiciones
fisiológicas como en determinadas condiciones patológicas, como la esclerosis lateral amiotrófica (Kawamata y Manfredi, 2010), las enfermedades de Alzheimer (Contreras et al, 2010) y Huntington (Browne, 2008; Ghandi et al, 2009) y el daño isquémico (Schinzel et al, 2005; Nakagawa et al, 2005).

3.2.1. Señalización por calcio intramitocondrial

Los iones de calcio pueden entrar a la matriz mitocondrial cuando su concentración es del orden de micromolar, en un transporte a través de un canal que sigue la cinética de un uniportador y que puede ser inhibido por rojo de rutenio y sus derivados (Gunter y Pfeiffer, 1990; Kirichok et al, 2004). Aunque las características básicas de este transporte se conocen desde hace muchos años, los componentes moleculares responsables sólo están siendo ahora identificados. Actualmente dos proteínas, MICU1 (mitochondrial calcium uptake 1) y MCU (mitochondrial calcium uniporter) se han descrito en el transporte de calcio hacia la mitocondria (Perocchi et al, 2010; Baughman et al, 2011; De Stefani et al, 2011). MICU1, una proteína de la membrana mitocondrial interna que posee un dominio transmembrana y dos dominios de manos EF, es probablemente el sensor de calcio de la maquinaria de transporte (Perocchi et al, 2010). Se identificó empleando la base de datos MitoCarta, un compendio de genes que codifican proteínas de localización mitocondrial (Pagliarini et al, 2008), y seleccionando genes candidatos que codificasen para proteínas de la membrana mitocondrial interna con expresión ubica y que estuvieran ausentes deSaccharomyces cerevisae (Carafoli y Lehninger, 1971), pero presentes en vertebrados y en cinetoplastidos (Benaim et al, 1990; Xiong et al, 1997). En cuanto a MCU, identificado tan sólo unos meses después por dos grupos independientes, es una proteína con dos dominios transmembrana, también localizada en la membrana mitocondrial interna, que se co-expresa con MICU1, y que se considera la formadora del canal para el transporte de calcio (Baughman et al, 2011; De Stefani et al, 2011).

Para mantener la homeostasis de calcio se requieren además mecanismos de eflujo de calcio en la mitocondria. Dos mecanismos de transporte se han descrito aunque, como en el caso del uniportador de calcio, la identidad de los genes y proteínas responsables sólo se está empezando a caracterizar ahora: el intercambiador Na+/Ca2+ y el intercambiador H+/Ca2+. El primero de ellos es abundante en tejidos excitable, como corazón cerebro y músculo esquelético, y cataliza el transporte con una estequiometría 3:2 (Gunter y Pfeiffer, 1990). Su identidad molecular se ha asignado recientemente a la proteína NCLX (Paltz et al, 2010). Por otro lado, el intercambiador H+/Ca2+ es abundante en cerebro, pero también en tejidos no excitable como hígado o pulmón, y el mecanismo de transporte es electroneutro, con dos protones intercambiados por cada ion de calcio (Gunter y Pfeiffer, 1990). Nuestro laboratorio purificó parcialmente de mitocondrias de hígado de rata dos proteínas de 66 y 55 kDa enriquecidas con actividad H+/Ca2+. Recientemente, LETM1, una proteína relacionada con el síndrome de Wolf-Hirschborn y previamente caracterizada como intercambiador K+/H+ de la membrana mitocondrial interna, se ha propuesto como responsable del transporte mitocondrial de H+/Ca2+ (Jiang et al, 2009). Estos autores
proponen que, a bajas concentraciones de calcio, la proteína actúa introduciendo iones de calcio en la matriz mitocondrial y extrayendo protones de forma sensible a rojo de rutenio, mientras que a concentraciones de calcio más altas, LETM1 catalizaría el eflujo de calcio mediante la entrada de protones a la matriz. Sin embargo, este trabajo es controvertido, ya que los resultados obtenidos no se ajustan a trabajos previos con LETM1 (Hajnóczky y Csordás, 2010).

Una vez en la matriz mitocondrial, el calcio promueve la activación de la respiración y la síntesis de ATP. Concentraciones de unos 4 nmoles de calcio/mg de proteína activan tres deshidrogenasas del ciclo de Krebs: la piruvato deshidrogenasa, la NAD-isocitrato deshidrogenasa y la oxoglutarato deshidrogenasa, que estimulan la generación de equivalentes reducidos (McCormack et al, 1990). En cardiomiocitos se ha descrito una activación por interacción directa entre el calcio y la ATP sintasa (Das y Harris, 1990).

3.2.2. Señalización por calcio extramitocondrial

Recientemente, un mecanismo mitocondrial de regulación por calcio, pero independiente de la entrada de calcio en la mitocondria, se ha descrito con la identificación de los CaMCs. Este grupo de MCs, que incluye a los AGCs y a los SCaMCs, se caracteriza por la presencia de dominios de manos EF orientados hacia el espacio intermembrana, donde unen calcio citósólico (Del Arco y Satrústegui, 2004; Fiermonte et al, 2004). La sensibilidad hacia el calcio de los dos tipos de proteínas es diferente, con los AGCs siendo más sensibles a señales de calcio más bajas (del orden de 100-350 nM) (Pardo et al, 2006; Contreras et al, 2007) y los SCaMCs activados por concentraciones de calcio más altas (del orden de 5-30 micromolar) (Caverio et al, 2005; Traba et al, 2008; Traba et al, 2012; Amigo et al, 2013), lo que correlaciona con las diferencias estructurales en los dominios de manos EF en ambos tipos de proteínas. Esta heterogeneidad en cuanto a la sensibilidad por calcio añade complejidad al mecanismo por el cuál las señales de calcio son transducidas a la mitocondria: señales bajas promueven la actividad de MAS mediante la activación de los AGCs, mientras que concentraciones más altas son reconocidas por los SCaMCs y el MCU. La activación mantenida del MCU inhibe tanto MAS (Contreras y Satrústegui, 2009; ver abajo) como la entrada de calcio en la mitocondria (Moreau y Parekh, 2008) y puede desencadenar la apertura del poro de permeabilidad de transición y la muerte celular (ver abajo).

- MAS y la señalización por calcio. La S0.5 para la activación por calcio de AGC1 y AGC2 es 280-350 nM y 100-150 nM respectivamente (Contreras et al, 2007). Por lo tanto, MAS puede ser activada mediante sus dominios de manos EF por señales de calcio que no producen la captación de calcio por la mitocondria (Palmieri et al, 2001; Pardo et al, 2006; Márímol et al, 2009). La activación de MAS induce la estimulación de la respiración mitocondrial por la transferencia de NADH a la matriz mitocondrial y evita la acumulación de lactato, que se produce en el citosol a partir del piruvato para regenerar NAD⁺ y mantener la glicolisis (Lasorsa et al, 2003).
A concentraciones más altas, el calcio puede entrar en la matriz mitocondrial a través del MCU y estimular la respiración por efecto directo sobre tres deshidrogenasas mitocondriales (ver arriba). En estas condiciones, en tejidos como corazón y cerebro, MAS se inhibe debido a que el sustrato común de ambas vías, el a-cetoglutarato, se convierte en limitante y se emplea de forma más efectiva por la a-cetoglutarato activada por calcio del ciclo de Krebs que por el transportador de oxoglutarato de MAS (Contreras et al, 2009). En hígado, sin embargo, este mecanismo regulador no se observa, probablemente porque el a-cetoglutarato no llega a ser limitante debido a su producción mediante la enzima glutamato deshidrogenasa, cuya activida es 5-9 veces mayor en este tejido que en cerebro o corazón (Contreras et al, 2009).

- **Transportadores de ATP-Mg/Pi y señalización por calcio.** En células respirando en condiciones normales, el gradiente de pH entre la matriz mitocondrial y el citoplasma favorece la captación de fosfato por la mitocondria a través del transportador de fosfato, mientras que la concentración de ATP es mayor en el citoplasma que en la mitocondria (Joyal y Aprille, 1992). Si una señal de calcio se produce en estas circunstancias, la activación del transportador de ATP-Mg/Pi produce la entrada de ATP-Mg o ADP, aumentando la concentración total de nucleótidos de adenina en la mitocondria (Aprille, 1993). Sin embargo, debido a la naturaleza no electrogénica del transporte, en situaciones donde el gradiente de pH se encuentra disminuido, o cuando las concentraciones de ATP-Mg o ADP son demasiado bajas, el transportador puede catalizar la reacción opuesta, resultando en un eflujo de nucleótidos de adenina desde la matriz al citoplasma (Aprille, 1988).

La alteración de los niveles de nucleótidos de adenina en la mitocondría podría tener consecuencias rmetabólicas relevantes. Vías o procesos con pasos dependientes de ATP dentro de la mitocondría, como la gluconeogénesis, el ciclo de urea, la síntesis de RNA y DNA, y el importe de proteínas, todas son susceptibles de estar reguladas por el contenido de nucleótidos de adenina (Aprille, 1988). El estado 3 respiratorio también es dependiente del contenido neto de nucleótidos de adenina (Asimakis y Aprille, 1980a), así como la capacidad de retención de calcio mitocondrial (Traba et al, 2012; Zoratti y Szabó, 1995 y referencias ahí incluidas).

### 3.2.3. El calcio y la transición de permeabilidad mitocondrial

Cuando la capacidad de tamponamiento de calcio mitocondrial se excede, un fenómeno denominado transición de permeabilidad (PT) tiene lugar. La PT se caracteriza por la pérdida de potencial de membrana mitocondrial, el hincharse de las mitocondrias y el eflujo de solutos de menos de 1500 Da, así como la liberación de factores pro-apoptóticos y, finalmente, la muerte celular (Halestrap, 2009). Aunque el calcio es la principal señal inductora de PT, se han descrito múltiples elementos reguladores que pueden aumentar o disminuir la susceptibilidad de la mitocondria a la PT, incluyendo el pH, los cationes divalentes, las especies reactivas de oxígeno y los nucleótidos de adenina (Rasola y Bernardi, 2011). La PT se ha descrito como importante en muchos paradigmas donde la sobrecarga de calcio juega un papel
importante, como en el infarto (Schinzel et al, 2005), la enfermedad de Parkinson (Ghandi et al, 2009) o la excitotoxicidad por glutamato (Li et al, 2009). El proceso de PT está mediado por la formación/activación de un canal no específico en la mitocondria denominado poro de transición de permeabilidad (PTP), cuyos componentes son desconocidos (Baines et al, 2009b). Entre las proteínas que se consideraban importantes en su estructura, tanto el ANT como el canal de aniones dependiente de voltaje se han descrito como no esenciales para la PT (Kokoszka et al, 2005; Baines et al, 2007). En cuanto a los elementos reguladores, la proteína más importante que ha sido identificada como moduladora de la apertura del PTP es la ciclofilina D (CypD), una peptidil prolil-isomerasa de la matriz mitocondrial cuya falta hace a las mitocondrias más resistentes a la PT (Baines et al, 2005; Nakagawa et al, 2005; Schinzel et al, 2005; Basso et al, 2005). El medio mediante el cual la CypD ejerce su modulación sobre la PT no está claro. Se ha sugerido que la ablación de CypD o su bloqueo farmacológico mediante ciclosporina A, desenmascara un sitio inhibitorio para fosfato, que en condiciones normales actúa como estimulador de la apertura del PTP (Basso et al, 2008). Sin embargo, otros laboratorios han sido incapaces de reproducir los experimentos que sostienen esta hipótesis (Varanyuwatana y Halestrap, 2012; McGee y Baines, 2012), por lo que el mecanismo de acción de la CypD sigue siendo desconocido.

4. FISIOLOGÍA Y PATOLOGÍA DE LOS TRANSPORTADORES MITOCONDRIALES DE ATP-Mg/Pi

Alterando el contenido neto de nucleótidos de adenina de la mitocondria, el transportador de ATP-Mg/Pi podría, en teoría, regular todas las reacciones de la matriz que requieren ADP y ATP (Aprille, 1988). Experimentos en los años 80 y 90 usando mitocondrias aisladas han sentado las bases de la reacción de transporte catalizada por el transportador y la correlación entre los niveles de nucleótidos de adenina mitocondriales y la actividad de reacciones dependientes de nucleótidos de adenina, como la carboxilación de piruvato (Aprille et al, 1981), la síntesis de citrulina (Goldstein and Aprille, 1982) y la síntesis de proteínas mitocondriales (Joyal et al, 1995). En años recientes, la identificación de los genes y proteínas de los SCaMCs ha permitido describir de forma más precisa las funciones conocidas del transportador, así como identificar otras nuevas.

4.1. Sal1p: el transportador mitocondrial de ATP-Mg/Pi en S. cerevisae

La levadura posee un único transportador mitocondrial de ATP-Mg/Pi, denominado Sal1p y que, como en el caso de mamíferos, se localiza en la membrana mitocondrial interna, tiene una estructura de seis dominios transmembrana y motivos de manos EF de unión a calcio en su N-terminal (Chen, 2004; Cavero et al, 2005). Sal1p también media el intercambio de ATP-Mg por fosfato inorgánico, aunque con requerimientos de calcio más altos que las formas de mamíferos (S0.5 de unos 30 mM, Cavero et al, 2005), comparado con los 5-15 mM de SCaMC-1 y SCaMC-3 (Traba et al, 2012; Amigo et al, 2013), probablemente debido a la presencia de sólo dos dominios de manos EF canónicos, mientras que las formas de mamíferos tienen cuatro (Cavero et al, 2005). Aunque la actividad de Sal1p no es
imprescindible para la levadura, se hace esencial en la ausencia de Aac2p, la forma principal del transportador de ATP/ADP en levadura, y la unión a calcio de Sal1p se ha demostrado como imprescindible en estas condiciones (Chen, 2004). Se ha propuesto que la letalidad por la falta de Sal1p y Aac2p se debe a la ausencia de un mecanismo de importe de ATP a la mitocondria, donde se requiere para la síntesis de proteínas (Cavero et al, 2005; Traba et al, 2009a; Traba et al, 2012). Recientemente, Kucejova y colaboradores han indicado que la pérdida de DNA mitocondrial podría ser la causa directa de la letalidad en las cepas deficientes en ambas proteínas, aunque los autores rechazan que se deba a un importe deficiente de ATP, ya que no observan diferencias en los niveles de nucleótidos en estas condiciones (Kucejova et al, 2008).

Sal1p es también la diapa de la señalización por calcio inducida por glucosa en la levadura. La unión de glucosa al receptor acoplado a proteínas G Gpr1p produce una señal de calcio citosólico que está mediada por la activación de PLC, la liberación de IP3 y la entrada de calcio desde el espacio extracelular (Ansari et al, 1999; Tisi et al, 2002; Tisi et al, 2004; Lemaire et al, 2004). Cavero y colaboradores han demostrado que, en ausencia de todos los parálogos del transportador de ATP/ADP (es decir, en condiciones donde Sal1p es indispensable), el efecto estimulatorio de la glucosa en la gemación desaparece completamente mediante el pretratamiento con EGTA, algo que no se observa en células salvajes o en cepas deficientes en Sal1p (Cavero et al, 2005; Granot y Snyder, 1993). Esta es la única diapa caracterizada hasta la fecha de este sistema de señalización por calcio inducida por glucosa.

4.2. SCaMC-2 y termogénesis

Recientemente, Anunciado-Koza y colaboradores han descrito un modelo de ratón deficiente en SCaMC-2. Este ratón es viable y no muestra un fenotipo aparente (Anunciado-Koza et al, 2011). Los autores habían previamente observado que SCaMC-2 se sobreexpresa en grasa inguinal y músculo esquelético de ratones deficientes en UCP1 y leptina, e hipotetizaron que la proteína podría ser importante para la termogénesis (Anunciado-Koza et al, 2011). Sus resultados usando ratones *SCaMC-2<sup>−/−</sup>* indican que su ausencia no afecta a la tolerancia a la glucosa, la resistencia a insulina o al mantenimiento de la temperatura corporal, pero los animales mutantes son resistentes a la obesidad inducida por la dieta y tienen una resistencia física más baja en cinta de correr. Además, fibroblastos embrionarios derivados de ratones *SCaMC-2<sup>−/−</sup>* presentan un flujo reducido de calcio desde el ER con bradiquinina, pero no con otros agonistas como taspigargina o ionimicina, y una metabolismo ligeramente glicolítico (Anunciado-Koza et al, 2011). La co-expresión de SCaMC-1 y SCaMC-3 en músculo esquelético, el tejido principal donde SCaMC-2 se expresa (Del Arco y Satrústegui, 2004), podría explicar la ausencia de un fenotipo más severo. Un efecto compensatorio tiene lugar de manera análogo en el ratón deficiente de UCP1, donde la expresión de UCP2 hace que los ratones no sean hiperfágicos ni predispuestos a la obesidad, como se había hipotetizado (Enerbäck et al, 1997).
Por otro lado, se ha sugerido que SCaMC-2 podría estar involucrado en la adaptación invernal en algunos tipos de peces, ya que su expresión en hígado se incrementa en respuesta a temperaturas bajas (Richards et al, 2008).

**4.3. SCaMC-1 y la retención de calcio mitocondrial**

El calcio es mantenido en la matriz mitocondrial por la formación de precipitados de calcio-fosfato, un proceso que requiere ATP (Carafoli et al, 1965; ver arriba). Por ello, mediante la alteración del contenido neto de nucleótidos, el transportador mitocondrial de ATP-Mg/Pi puede regular la susceptibilidad de las mitocondrias a la PT. Hace algunos años, Hagen y colaboradores propusieron que parte del efecto del fosfato como inductor de PT podría deberse al eflujo de nucleótidos de adenina a través del transportador mitocondrial de ATP-Mg/Pi activado por calcio (Hagen et al, 2003). Además, se ha observado que SCaMC-1 está sobreexpresado en cánceres de mama (Chen et al, 2011), sugiriendo un posible papel de la proteína en la resistencia frente a estímulos de muerte observada en células transformadas (Hanahan y Weinberg, 2011). Recientemente se ha obtenido una comprobación directa de este fenómeno por Traba y colaboradores, que han demostrado que niveles altos de SCaMC-1 correlacionan positivamente con resistencia a estímulos que inducen muerte celular por sobrecarga de calcio, mientras que el silenciamiento de la proteína reduce la retención mitocondrial de calcio y favorece la muerte celular (Traba et al, 2012).

**4.4. El transportador mitocondrial de ATP-Mg/Pi y el metabolismo hepático**

El tejido donde más a fondo se ha estudiado el transportador mitocondrial de ATP-Mg/Pi es el hígado de rata. También fue aquí donde se observó por primera vez su actividad, describiéndose como importante en el llenado de nucleótidos que tiene lugar en las mitocondrias de hígado de mamíferos durante la primera hora postnatal (Sutton y Pollak, 1978; Sutton y Pollak, 1980; Aprille y Asimakis 1980). Dos señales complementarias se han propuesto como mediadoras de este proceso. Primero, el aumento de los niveles de ATP citósólico y del ratio ATP/ADP resultantes de la liberación de hormonas que promueven la glicólisis y la glucógenolisis, como el glucagon y la adrenalina, que se secretan poco después del nacimiento (Sutton y Pollak, 1980; Cuezva et al, 1982). En segundo lugar, el hinchamiento de los pulmones durante el nacimiento, que provoca una perfusión del hígado con sangre oxigenada y permite el funcionamiento de la cadena transportadora de electrones y la generación de un gradiente de protones mitocondrial (Aprille, 1990). La formación del gradiente de protones permite la entrada de fosfato en la mitocondria a través del transportador de fosfato, que puede ser a continuación intercambiado por ATP citósólico a través del transportador de ATP-Mg/Pi activado por las señales de calcio producidas por las hormonas (Aprille, 1993).

De acuerdo con esto, el transportador de ATP-Mg/Pi se ha propuesto como una diana del glucagon y otros agentes mobilizadores de calcio, ya que se ha observado que el contenido neto de nucleótidos de adenina
en las mitocondrias de hígado aumenta en respuesta a estas señales (Bryla et al, 1977; Titheradge et al, 1979; Titheradge y Haynes, 1980; Aprille et al, 1987), y que la entrada de nucleótidos de adenina es inhibida por agentes quelantes de calcio (Haynes et al, 1986). Sin embargo, se ha propuesto también un mecanismo de entrada de nucleótidos independiente del transportador de ATP-Mg/Pi, según el cuál la entrada de calcio en la mitocondria inhibiría la pirofosfatasa mitocondrial, promoviendo la acumulación de pirofosfato intramitocondrial, que a continuación podría ser intercambiado por nucleótidos de adenina citósicos a través del ANT (Davidson y Halestrap, 1988).

Además, Aprille y colaboradores han descrito correlaciones entre los niveles de nucleótidos de adenina mitocondriales y actividades metabólicas dependientes de ellos en la matriz mitocondrial, tal y como la carboxilación de piruvato (Aprille et al, 1981) y la síntesis de citrulina (Goldstein y Aprille, 1982), que son pasos importantes de la gluconeogénesis y la síntesis de urea, respectivamente.
OBJECTIVES
The main goal of the present work is to characterise the role of SCaMC-3, the main paralog of the ATP-Mg/Pi carrier present in liver and brain, both in physiological and pathological conditions. As for the first, the carrier has been described as a putative target of glucagon signalling in liver mitochondria important in the regulation of gluconeogenesis and ureogenesis. The carrier has also been implicated in the filling of adenine nucleotides that takes place in liver mitochondria during the first post-natal hours. Regarding the role of SCaMC-3 in cell death, recent work has implicated the ATP-Mg/Pi carrier in the regulation of calcium retention in mitochondria, a critical step in cell signalling whose defects are linked with several pathological conditions, including ischemia/reperfusion and excitotoxicity. Therefore, we have set three main objectives for the present work:

1. **Characterization of the general features of SCaMC-3/− mice and SCaMC-3-deficient mitochondria.**
   a) Analysis of the different paralogs of the ATP-Mg/Pi carrier in different tissues and developmental stages.
   b) Effect of the lack of SCaMC-3 in the levels of glucose and urea and its response to fasting.
   c) Evaluation of putative functional defects in mitochondrial respiration due to absence of SCaMC-3.

2. **Role of SCaMC-3 in respiration from liver mitochondria in response to glucagon.**
   a) Role of SCaMC-3 in oxidative phosphorylation in mitochondria depleted from adenine nucleotides.
   b) Effect of glucagon in vivo in liver mitochondrial adenine nucleotide levels, respiration and mitochondrial calcium retention capacity and in vitro, using hepatocytes lacking SCaMC-3.

3. **Analysis of the effect of the absence of SCaMC-3 in calcium-overload models in brain.**
   a) Characterization of the expression of SCaMC-3 in brain.
   b) Effect of the absence of SCaMC-3 in kainate-induced injury.
   c) Effect of the absence of SCaMC-3 in in vivo and in vitro models of ischemia/reperfusion.
MATERIALS AND METHODS
1. In vivo experiments

1.1. Generation of SCaMC-3−/− mice

Mice deficient in SCaMC-3 were generated by Lexicon Pharmaceuticals by homologous recombination (Figure 2.1). A DNA disruption cassette, with exons 2 and 3 substituted by a 5’-SCaMC-3-lacZ-Neo, was transfected into 129SvEvBrd (Lex2) ES cells. ES SCaMC-3−/− cells were injected into blastocysts to generate chimeric mice containing the disruption cassette in the germinal line. These mice were further bred into heterozygous SCaMC-3−/+ and homozygous SCaMC-3−/−. For the maintenance of the colony, and to avoid consanguinity, heterozygous SCaMC-3−/+ were bred with Svj129xC57BL/6 mice (Harlan Iberica). Mice were housed with a 12-hour light cycle and fed ad libitum on standard chow. All animal work performed in this study was carried out in accordance with procedures approved in the Directive 86/609/EEC of the European Union with approval of the Ethics Committee of the Universidad Autónoma de Madrid.

1.2. Genotyping

To assay the genotypic identity of the mice, genomic DNA was extracted from the tails of the mice using an extraction kit (Roche) and genotyping was performed by double PCR using the primers shown in Table 2.1. The PCR program starts with an initial heating of 5 minutes at 94°C, followed by 30 cycles as follows: 1 minute at 94°C, 30 seconds at 58°C and 30 seconds at 72°C. After the 30 cycles, a final extension stage is performed for 5 minutes at 72°C. PCR products are resolved by electrophoresis in 1.5 % agarose gels and identified by their fragment sizes, with a 206 bp fragment corresponding to the wild type allele and a 354 bp to the mutated allele (Figure 2.1).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (forward)</td>
<td>5′-TGAGGCAATGAGGCATATTCTA-3′</td>
<td>Wild type</td>
</tr>
<tr>
<td>10 (reverse)</td>
<td>5′-AAGGCTGTGAACATGAGCA-3′</td>
<td>Wild type</td>
</tr>
<tr>
<td>Neo3a (forward)</td>
<td>5′-GCAGCGCATCGCTTACCTGCTAC-3′</td>
<td>Knock-out</td>
</tr>
<tr>
<td>12 (reverse)</td>
<td>5′-GGGTAGCTGATTACGTGCACGTC-3′</td>
<td>Knock-out</td>
</tr>
</tbody>
</table>

Table 2.1. DNA primers used for genotyping. See Figure 2.1 for details on the location of the sequences recognized by each primer and the sizes of the fragments obtained for each genotype.

1.3. Glucose and urea measurements

Glucose and urea blood levels were measured in 3-month old wild type and SCaMC-3−/− mice before and after a 24 or a 48-hour fasting (n=5 for each genotype and time point, except for glucose measurements after 48 hour-fasting, where n=10 for each genotype). Blood was collected from the submandibular vein using a lancet. Glucose was measured from a drop of blood using Accutrend stripes and a glucose reader (Roche), whereas for urea measurements, serum was obtained by centrifugation of the blood (700g, 5 min) and quantification performed with a detection kit (Spinreact) that measures urea levels by coupling its hydrolysis by urease with NADH-dependent reduction of α-ketoglutarate to glutamate.
Figure 2.1. Genotyping strategy by PCR. A. Genetic structure of the SCaMC-3 locus. Exons are numbered from 1 to 10. B. Localization of the sequences from the wild type allele and the targeted allele, recognized by the primers used in the PCR reaction. The sequences of the primers (named 9, 10, 12 and Neo3a) are displayed in Table 2.1. C. PCR products after double PCR using the indicated primers. Animals containing two copies of the wild type allele show a single band of 206 bp, while animals containing two copies of the targeted allele show a single band of 345 bp. Heterozygous animals display both bands.

1.4. Glucagon administration and mitochondrial adenine nucleotide quantification by HPLC

Wild type and SCaMC-3-/- mice (n=5-6 animals for each group) were injected intraperitoneally with 2 mg/kg glucagon (Sigma-Aldrich) in 0.9% NaCl or vehicle and sacrificed by cervical dislocation 15 minutes after administration. Liver mitochondria were then extracted using a fast protocol to prevent adenine nucleotide loss. Liver was homogenized using the following extraction buffer: 250 mM sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4. The isolation procedure to obtain a mitochondrial-enriched fraction consisted of a single low-speed centrifugation (700g, 10 minutes) followed by a high-speed centrifugation (10,000g, 15 minutes) of the resulting supernatant. The pellet obtained after the second centrifugation was re-suspended in 660 mM HClO₄ and kept at -80°C until further processing.

Quantification of the adenine nucleotide content was performed by HPLC in collaboration with Dr. Maria del Mar González Barroso (CIB, CSIC) as previously described (Vives-Bauza et al, 2007). HClO₄-treated mitochondrial fractions obtained as described above were neutralized using K₂CO₃, kept on ice 10 minutes and then maintained at -80°C for one hour to allow decant of the perchlorate precipitate. Samples were then centrifuged (5000g, 10 minutes) and 50 mL of the supernatant were injected in the column and monitored by UV at 210 nm from 15 to 45 minutes. To asses the identity of each peak and quantify the amount of the species of interest, standard curves were constructed by plotting peak heights versus known concentrations of ATP, ADP and AMP.

1.5. Liver and brain sections

To obtain liver and brain sections, wild type and SCaMC-3-/- mice were anaesthetised using 0.5 mg/g chloral hydrate and perfused transcardially using 0.9% NaCl for 10 minutes followed by PFA 4% for another 10 minutes. Livers or brains were removed and postfixed overnight in PFA 4%. In the case of liver, samples were then transferred to a phosphate buffer solution and embedded in paraffin. Liver coronal sections of 5 µm were obtained using a cryotome an mounted in polylysine-coated glass slides (Menzel-
Glässer). Livers that were not immediately sectioned were kept in 70% ethanol at 4°C. Paraffin embedding and tissue sectioning were performed by the histology service of the Centro Nacional de Biotecnología (CSIC, Madrid). Conversely, brains were transferred to a phosphate buffer solution containing 30% sucrose after post-fixation, and left 36-48 hours, until sank to the bottom of the container, after which were embedded in OCT compound (Tissue-Tek), frozen on dry ice and cut into 8 series of 40 μm coronal sections with a cryotome (free-floating sections). Sections were kept at 4°C in PBS for short-term periods, or in a cryoprotectant medium (25% glycerol, 25% ethylene glycol in 50 mM phosphate buffer) at -20°C for longer storage.

1.6. Hematoxylin/eosin staining

Liver and brain sections were stained with hematoxylin and eosin to analyse their structure. Hematoxylin colours the nuclei of the cells in blue, while eosin dyes other structures with different shades of red (Resaz et al, 2011). In the case of liver, sections were deparaffinized and hydrated before staining by sequential immersion in xylol (2 minutes), absolute ethanol (1 minute), 96% ethanol (1 minute), 70% ethanol (1 minute) and distilled water. As for brain, sections were mounted in polylysine-coated glass slides (Menzel-Glässer) prior to staining. Slides were then stained with Mayer's hematoxylin (50 g/L aluminium potassium sulphate, 1 g/L hematoxylin, 0.2 g/L sodium iodate and 20 mL/L glacial acetic acid) for 5 minutes, washed in distilled water and incubated in 0.25% eosin (Sigma-Aldrich) for 5 minutes. Excess of dye was removed using 70% ethanol. Sections were then dehydrated in 96% ethanol (3 minutes), 100% ethanol (3 minutes) and xylol (3 minutes), and mounted using DPX (VWR International Ltd).

After staining, Images were taken using a 5x objective in an Axioskop2 vertical microscope (Zeiss) coupled to a Coolscan FX CCD camera (Roper scientific). Individual 5x images were merged using Photoshop (Adobe) to obtain high resolution images of larger areas.

1.7. Transient cerebral ischemia, determination of neurological scores and infarct volume determination.

Surgery to occlude the middle cerebral artery was performed by Xavier De La Rosa, from Anna Planas' laboratory (IDIBAPS, Barcelona) as previously described (Pérez-Asensio et al, 2010). Wild type and ScAMC-3/3- 3-month old male mice (n=4-7) were anaesthetized with 2% isoflurane vaporized in O2 and N2O (30:70). Focal cerebral ischemia was achieved by 60 or 90 minutes intraluminal occlusion of the right middle cerebral artery, followed by reperfusion. Cerebral blood flow was monitored throughout the surgery using a Laser-Doppler probe. Mice with a flux drop during occlusion inferior to 60% and mice that did not recover baseline fluxes after reperfusion were discarded. 48 hours after reperfusion, mice were anaesthetized with isoflurane and perfused transcardially using saline buffer (0.9% NaCl) containing heparin (1mL/L). Brains were extracted, frozen in -40°C isopentane cooled in dry ice and maintained at -80°C until further processing.
Neurological scores after cerebral ischemia were determined for each animal 24 and 48 hours after surgery to evaluate the effect of artery occlusion on movement and coordination, as previously described (De Simoni et al, 2003). Five different parameters were studied: body symmetry, gait, circling behaviour, front limb symmetry and compulsory circling. To each one, a value from 0 to 4 was assigned in a blind manner depending on the severity of the phenotype, with 0 being the less severe and 4 the most affected. Final score resulted from the addition of the five individual values and therefore ranges 0 to 20.

Non-fixed brains from arterial-occluded mice were embedded in OCT compound (Tissue-Tek) and cut into 10 series of 20 μm sections. Every series contained 6 sections, distanced 1 mm between them. Series were kept at -20°C until used. Slides were dried using a warm air flux before fixing the tissue in PFA for 15 minutes. Excess of PFA was washed in distilled water before staining 1 hour with cresyl violet (0.1% in water, with a few drops of 10% acetic acid). Sections were then dehydrated in 96% ethanol (3 minutes), 100% ethanol (3 minutes) and xylol (3 minutes), and mounted using DPX (VWR International Ltd). To evaluate the extent of the infarct, sections were photographed using a Leica MZ6 magnifying glass attached to a Canon EOS 450D digital reflex camera, and infarct areas were determined in a blind manner using ImageJ software. Infarct volume was calculated integrating the different areas measured. To avoid overestimation of the infarct volume as a result of the presence of oedema, measures in the ipsilateral hemisphere were normalized with contralateral areas. Infarct volume was expressed as a percentage of ipsilateral volume.

1.8. Kainic acid treatment and determination of astrogliosis and cell death

25 mg/kg kainic acid (KA; Sigma-Aldrich) was administrated intraperitoneally to 3-month old male wild type and SCaMC-3/− mice (n=8 mice for each genotype). In each experiment, wild type and SCaMC-3/− mice from the same litter were visually monitored in parallel for two hours and seizure severity was quantified in a blind manner using the following classification (McLin and Steward, 2006): 1. Unmoving and crouched in a corner, staring; 2. Stretched body out, tail becomes straight and rigid, ears laid back, bulging eyes; 3. Repetitive head bobbing, rears into a sitting position; 4. Rearing and falling, tonic clonic seizures broken by periods of total stillness, jumping clonus, running clonus; 5. Continuous level 4 seizures; 6. Body in clonus, no longer using limbs to maintain posture, usually precursor to death; 7. Death. For data analysis, the most severe state every 5 minutes was used. Mice were sacrificed seven days after drug administration and brain sections extracted for histological analysis as described above.

To evaluate astrogliosis, fluorescent immunohistochemistry detection of GFAP was performed. Free-floating brain sections from KA-treated mice were washed in PBS and blocked in PBS-1% Horse serum-0.25% Triton X-100 for one hour before incubating overnight with the primary antibody (GFAP, Danko, 1:200) in the same medium at 4°C. Excess of antibody was washed three times in PBS and a secondary
fluorescent antibody (Alexa 488 α-rabbit; Invitrogen, 1:250) was then incubated for 1 hour. Secondary antibody was washed three times in PBS and sections were mounted in polylysine-coated glass slides (Menzel-Glässer.) using mowiol. Immunoreactivity was quantified using images of three different coronal sections of the hippocampus, corresponding approximately to positions 261, 281 and 301 from Allen Brain Mouse Atlas (Lein et al, 2007), were taken at 10x with an Axioskopplus2 vertical microscope (Zeiss) coupled to a Coolsnap FX CCD camera (Roper Scientific) using the same settings for all the samples. GFAP fluorescence was quantified using ImageJ software. A region of interest including CA1, CA2, CA3 and the dentate gyrus was delimited and the total area within calculated. Total area occupied by GFAP fluorescence was quantified by manually introducing a threshold which was maintained throughout the analysis of all images. Measures were performed in a blind manner. The result is shown as the percentage of GFAP fluorescence respect to the total area.

Degenerating neurons in brain sections from KA-treated mice were identified using Fluoro-Jade B staining (Histo-Chem Inc) (Schmued et al, 1997). Brain sections mounted in polylysine-coated slides were briefly rinsed in distilled water and incubated sequentially in 100% ethanol (3 minutes), 70% ethanol (1 minute), 30% ethanol (1 minute) and distilled water (1 minute). Sections were then transferred to 0.06% KMnO₄ for 15 minutes, and excess of potassium permanganate was removed in distilled water. Slides were incubated in 0.001% Fluoro-Jade B for 30 minutes in the dark, rinsed in distilled water and left to dry before washed in xylol and mounted using DPX.

2. Experiments using isolated mitochondria

2.1. Mitochondria isolation

Mice were sacrificed by cervical dislocation and brains and/or livers were removed and minced in extraction buffer (250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 25 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.1% BSA and protease inhibitors 1mM PMSF and 1mM iodoacetamide). Isolation of the mitochondrial fraction was performed using differential centrifugation as previously described (Frezza et al, 2007). Tissue was homogenized using a Dounce homogenizer (10 passes with a loose pestle and 10 passes with a tight pestle) and the resulting homogenate was centrifuged at low speed (700g, 10 minutes). The pellet containing nuclei and intact cells was discarded, and the supernatant was centrifuged again in the same conditions (700g, 10 minutes). The resulting supernatant was centrifuged at higher speed (10,000g, 15 minutes) to obtain a pellet containing the mitochondrial fraction. The pellet was resuspended in MSK buffer (75 mM D-mannitol, 25 mM sucrose, 5 mM KH₂PO₄, 20mM Tris-HCl, 0.5 mM EDTA, 100 mM KCl and 0.1% BSA free of fatty acids) and centrifuged again at high speed (10,000g, 15 minutes). Finally, the resulting pellet was resuspended in MSK and the amount of protein was quantified by the Bradford assay (Bio-Rad), using BSA to build a standard curve.
2.2. Mitochondrial adenine nucleotide depletion and filling

Mitochondrial samples were depleted from adenine nucleotides by incubation in MSK with 2 mM tetraysodium pyrophosphate for 5 minutes at 30°C (Aprille and Asimakis, 1980; D'Souza and Wilson, 1982). Subsequently, mitochondria were pelleted by centrifugation (10,000g, 5 minutes) and resuspended in fresh MSK. In every case, a sample was used to confirm adenine nucleotide depletion by measuring oxygen consumption in the presence of 1 mM EGTA, as depleted mitochondria are unable to stimulate respiration after ADP addition (Henke and Nickel, 1992). To refill mitochondria with ATP-Mg, adenine nucleotide-depleted mitochondria were resuspended in MSK devoid of EDTA and supplemented with 2 mM ATP, 200 nM ruthenium red and 5 mM MgCl₂, at 30°C, for 1, 2, 3 and 10 minutes. Exogenous calcium was not added during the filling reaction, as impurities in MgCl₂ are enough to activate the ATP-Mg/Pi carrier (Nosek et al, 1990). Incubations were stopped by introducing the sample tubes on ice, centrifuged (10,000g, 5 minutes) and resuspended in MSK supplemented with 1 mM EGTA for oxygen consumption measurements.

2.3. Oxygen consumption measurements using Clark electrode

Mitochondrial respiration rates were determined using a Clark oxygen electrode (Hansatech Instruments Ltd). The electrode was calibrated using sodium dithionite to obtain a zero oxygen line. Measurements were performed in MSK medium supplemented with 1 mM EGTA except when indicated, using 0.1-0.3 mg/protein from the mitochondrial fraction. When brain mitochondria were used, 100 μM digitonin was added to release mitochondria contained in synaptosomes (Jalil et al, 2005). State 4 respiration (i.e. in the absence of ADP) was assayed using 5 mM glutamate plus malate (complex I), or 2 μM rotenone plus 5 mM succinate (complex II). State 3 (i.e. in the presence of ADP) was induced by addition of 0.5 mM ADP, unless noted otherwise. Uncouple respiratory rates were obtained using 1-5 μM FCCP (when succinate or TMPD plus ascorbate were used as substrates) or 80 μM dinitrophenol (when substrates were glutamate plus malate). When oligomycin was used to inhibit ATP synthase, it was added at a concentration of 0.2 mg/mL. Respiratory control rates were obtained by dividing state 3 rates by state 4 rates. Basal rates were subtracted from all measures.

2.4. Citrate synthase activity

Mitochondrial mass from liver and brain was inferred by measuring citrate synthase activity in isolated mitochondria from these tissues as previously described (Barrientos, 2002). In this reaction, the production of citrate from acetyl-CoA and oxaloacetate by means of the matrix enzyme citrate synthase is coupled to the conversion of dithionitrobenzoic acid (DTNB) to thionitrobenzoic acid (TNB), which has intense absorption at 412 nm. Mitochondria were isolated as described above and stored at -80°C. At the time of use, mitochondria were diluted to a final concentration of ~100 (brain) and ~200 (liver) mg/mL in a reaction medium containing 0.1 mM DTNB, 0.25% Triton X-100, 0.5 mM oxaloacetate and 0.3 mM acetyl-CoA. Measurements were performed in a Thermo Electron Corporation spectrophotometer at 30°C.
with a stirrer, and the rate of the reaction was calculated as the slope of the linear change in absorption. Conversion to enzyme activity was achieved using the Lambert-Beer equation taking 13.6 mM⁻¹ cm⁻¹ as the value of molar extinction coefficient for TNB (Ellman, 1958).

2.5. Calcium uptake measurements

The calcium retention capacity (CRC) of isolated liver mitochondria was measured with 0.1 μM Calcium Green-5N as an extra-mitochondrial calcium indicator in MSK devoid of EDTA and supplemented with 1 mM MgCl₂ as previously described (Traba et al, 2012). An Aminco-Bowman (series 2) fluorimeter provided with temperature control and continuous stirring was used. All experiments were carried out at 30°C in the presence of respiratory substrates (5 mM succinate plus 2 μM rotenone) and in the presence or absence of adenine nuucleotides (ATP or ADP). After 3-5 min of incubation, mitochondria were challenged with subsequent 10-20 nmol CaCl₂ additions as indicated in the figure legends, and calcium uptake into mitochondria was measured as a decrease in fluorescence. The CRC was determined as the total amount of calcium (in nmol per mg of protein) that mitochondria are able to take up before starting to release it to the extra-mitochondrial medium.

3. In vitro experiments

3.1. Primary cultures

3.1.1. Primary cultures of cortical neurons and cortical astrocytes

For isolation of primary cortical neurons, homozygous crosses were performed to obtain wild type or SCamC-3⁻/⁻ embryos. At embryonic day 16, pregnant females were sacrificed and embryos extracted. Cerebral cortex was dissected from embryonic brains and incubated with papain (Roche) in PBS enriched with 1% BSA (PBS-B) for 15 minutes in a 37°C water bath shaker. After the 15 minutes, a volume of PBS-B supplemented with 100 U/mL DNase (Roche) was added to stop the trypsin reaction, and cortex were gently dissociated using a plastic pipette. Medium was removed by centrifugation, fresh PBS-BSA with DNase was added and tissue was further dissociated using a flame polished Pasteur pipette. Cells that had not been dissociated were left to decant for 5 minutes, while the rest of the medium was transferred to a new tube, centrifuged and the pellet resuspended in a 1:1 solution of Horse serum and Neurobasal medium supplemented with B27, Glutamax and gentomycin (all from Gibco) at the concentrations indicated by the manufacturer. After counting, cells were plated at a density of 1.5 x 10⁵ cells/mL and medium was replaced after 4-6 hours by complete Neurobasal medium supplemented with B27, Glutamax and gentomycin, but without serum. For cell maintenance, cell medium was partially (~50%) changed by fresh medium every 2-3 days. Experiments were performed after 10-11 days in vitro.

Cultures of primary astrocytes were obtained similar to cortical neurons, except that cortices were obtained from wild type SCamC-3⁻/⁻ pups from postnatal ages 0 to 3 and, after the isolation procedure, the final pellet of cells was resuspended in Dulbecco's modified Eagle medium (DMEM) (Gibco)
supplemented with 10% Foetal bovine serum (Gibco) supplemented with Glutamax and a gentomycin. Culture medium was replaced completely every 2-3 days. Experiments were performed after 14-15 days in vitro.

3.1.2. Primary cultures of hepatocytes
Isolation of primary mouse hepatocytes was performed in collaboration with Drs. Aránzazu Sánchez and Margarita Fernández at the Universidad Complutense. Livers from 12-24 hour-fasted animals were perfused with Hank's balanced salt solution supplemented with 10 mM Heps and 0.2 mM EGTA for 5 minutes, followed by a longer perfusion of 10-15 minutes with William's medium E containing 10 mM Heps and 0.03% collagenase H (0.19 U/mg). Liver was then minced with scissors and viable hepatocytes selected by isodensity centrifugation in Percoll and seeded in DMEM:F12 (1:1) (Gibco) at a density of 0.6 x 10^6 cells/cm² in collagen I-coated XF24 V7 cell culture plates (Seahorse Bioscience) (area 0.32 cm²) or in collagen I-coated coverslips in p24 wells (area 1.9 cm²). Cell were kept overnight at 37°C and 5% CO₂, and used 12-16 hours later.

3.2. X-Gal staining and immunocytochemistry
The disruption cassette used to generate SCaMC-3−/− mice includes the lacZ gene, which codes for the b-galactosidase enzyme from Escherichia coli. Activity of this enzyme in transgenic mice can be monitored through the use of a chromogenic substrate, such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), to infer the expression of the original locus (Cui et al, 1994). Detection of β-galactosidase in cells from SCaMC-3-deficient cultures was performed using an X-Gal staining kit (Stratagene) as previously described (Contreras et al, 2010b). Cells were fixed in 4% PFA for 15 minutes and incubated for 48 hours at 30°C in X-Gal solution, after which immunocytochemistry detection was performed. Cells were incubated in PBS-0.25% Triton X-100-5% BSA for one hour for permeabilization and blocking. Incubation of the cells with primary antibodies was done using the same solution, and left overnight at 4°C. Monoclonal antibody against neuronal marker NeuN (Chemicon International) was used at 1:100, whereas polyclonal antibody against astrocytic marker IGFAP antibody (Dako) was used at 1:500. Washes using PBS were performed to remove excess of primary antibodies before incubation for 1 hour with secondary fluorescent antibodies (Cy3 α-mouse (1:1200), Cy3 α-rabbit (1:1200), FITC α-mouse (1:250) or Alexa 488 α-rabbit (1:250), (Invitrogen). Excess of secondary antibodies was washed in PBS, and 1 μg/mL DAPI (Calbiochem) was added for 10 minutes to dye the nuclei.

X-Gal staining was also performed in free-floating brain sections by incubation in X-Gal solution for 48 hours at 30°C. Sections were then mounted on polylysine-coated glass slides (Menzel-Glässer) and counter stained with hematoxylin/eosin as indicated above.

3.3 Western blot
For protein expression analysis by western blot, tissue homogenates or cellular pellets were collected
either in extraction buffer (50 mM sucrose, 1 mM EDTA, 1 mM EGTA, 25 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.1% BSA and protease inhibitors 1mM PMSF and 1mM iodoacetamide) or in RIPA buffer respectively, disrupted by sonication and quantified using the Bradford protein assay solution (Bio-Rad). Samples that were not used immediately were kept at -70°C. Loading buffer (50 mM Tris-HCl, 100 mM DTT, 2% SDS, 1.5 mM bromophenol blue and 10% glycerol) was added to the samples (10-50 μg) before boiling them for 5 minutes. Samples were then resolved in SDS-polyacrilamide gel electrophoresis (SDS-PAGE), using 10% polyacrylamide gels (Bio-Rad). Samples contained in the gels were transferred to a nitrocellulose membrane of 0.2 μm pore size (Whatman) by electrical means (200 mA, 2 hours, RT, or 30V, overnight, 4°C), using an appropriate buffer (20 mM Tris, 150 mM glycine, 20% methanol and 0.2% SDS). Membranes were stained with 2% Ponceau red to confirm protein transfer, and then blocked in Tris-buffered saline (TBS: 150 mM NaCl, 100 mM Tris-HCl, pH 7.5) containing 0.1% Tween-20 and 5% non fat dry milk for 2 hours at room temperature, or overnight at 4°C. Membranes were then incubated with primary antibodies in blocking solution (2 hours at room temperature or overnight at 4°C) and washed in TBS-0.1% Tween 20 before incubation in blocking solution with secondary antibodies coupled to peroxidase (HAM-PO for mouse monoclonal primary antibodies, or GAR-PO for rabbit polyclonal antibodies). Membranes were further washed in TBS-Tween 20 0.1% and developed using ECL (Perkin-Elmer).

Monoclonal antibodies MAP2 and Hsp60 were purchased at Sigma and used at a dilution of 1:2,500 and 1:10,000 respectively, whereas polyclonal GFAP (Dako) was used at 1:2,500. Rabbit polyclonal antibodies generated in our laboratory against the N-terminus of the proteins SCaMC-1 and SCaMC-3 (Del Arco and Satrústegui, 2004) were used at a dilution of 1:5000. Polyclonal antibody against SCaMC-2 (Sigma-Aldrich) was used at 1:2,000. Polyclonal β-ATPase antibody was a kind gift of Dr. José Manuel Cuezva and was used at a dilution of 1:10,000.

3.4. Calcium measurements

To monitor cytosolic calcium changes, hepatocytes growing on coverslips were loaded with 5 μM Fura-2-AM (Molecular Probes) for 15 minutes at 37°C in calcium-free HCSS medium (120 mM NaCl, 0.8 mM MgCl₂, 25 mM Hepes, 5.4 mM KCl) supplemented with 0.05% pluronic acid F-127 (Invitrogen) and washed for 20 minutes at 37°C in HCSS with 2 mM CaCl₂. Coverslips were then mounted in the perfusion chamber of a Zeiss microscope as previously described (Mármol et al, 2009), and fluorescence was imaged ratiometrically using alternate excitation at 340 and 380 nm and a 510-nm emission filter with a Neofluar 40X/0.75 objective at 37°C. Excitation at 340 nm is only absorbed by calcium-bound Fura-2-AM, whereas excitation at 380 nm is absorbed by calcium-free Fura-2-AM (Grynkiewicz et al, 1985). Agonists were added as a bolus. Image acquisition and analysis were performed using Aquacosmos 2.6.2.4 software (Hamamatsu). Ratio signals (fluorescence exciting at 340 nm divided by fluorescence exciting at
380 nm) were converted to calcium concentrations using the formula \([\text{Ca}^{2+}] = Kd \times \text{b} \times (\text{R} - \text{Rmin})/(\text{Rmax} - \text{R})\) described in (Gryniewicz et al., 1985), where \(\text{R}\) is the fluorescence ratio at each time point, while \(\text{Rmax}\) and \(\text{Rmin}\) are determined by measuring the ratio after addition of ionophore A23187 to cells in HCSS medium with 2 mM CaCl\(_2\) (Rmax) or with 1 mM EGTA (Rmin). For \(\text{Rmax}\) and \(\text{Rmin}\) calculations, autofluorescence of the cells at each wavelength was obtained by adding 4 mM MnCl\(_2\) to fully chelate the fluorescence of the probe, and subtracted. The value for \(\text{Kd}\) (i.e. effective dissociation constant of Fura-2-AM for calcium) was assumed to be 224 mM (Gryniewicz et al., 1985) and \(\text{b}\) was calculated by dividing the maximum and the minimum fluorescences at 380 nm (Gryniewicz et al., 1985).

### 3.5. Oxygen consumption measurements in primary hepatocytes

To assess respiratory parameters in primary hepatocytes, a Seahorse Bioscience XF24-3 Analyzer (Seahorse Bioscience) was used as previously described (Gómez-Galán et al., 2011). The Seahorse XF Analyzer measures oxygen concentration in a small volume of medium above cells growing in a monolayer, which allows for real-time measurement of bioenergetic parameters in attached cells (Seahorse Bioscience website, http://www.seahorsebio.com/products/how-xf-works.php). To assess these parameters, four injectors (A-D) sequentially add different compounds that affect the bioenergetic status of the cell (Brand and Nicholls, 2011). In our case, injector A was loaded with either glucagon (final concentration: 0.1 \(\mu\)M), phenylephrine (final concentration: 100 \(\mu\)M) or vehicle (DMEM). Injector B was loaded with oligomycin (final concentration: 6 \(\mu\)M), which inhibits the ATP synthase and, therefore, coupled respiration. Injector C was loaded with FCCP (final concentration: 1 \(\mu\)M), that dissipates the proton gradient and promotes maximum mitochondrial respiratory rates. Finally, injector D was loaded with rotenone and antimycin A (final concentration: 1 \(\mu\)M each), which inhibit the electron transport chain at complexes I and III respectively, blocking mitochondrial respiration and allowing to obtain the value of oxygen consumption rate which is due to non-mitochondrial respiration. An outline of the experimental design is represented in Figure 3.9A.

Culture medium of the cells was replaced before the measure with DMEM:F12 devoid of bicarbonate and supplemented with 10 mM sodium lactate (Sigma-Aldrich), with a final glucose concentration of 5 mM and kept at 37°C for 1 hour. Four wells were used for each condition (vehicle, glucagon and phenylephrine). Four measurements were performed before the first addition and three after the addition of each compound and the average was used for calculations.

The effects of vehicle, glucagon and phenylephrine were calculated in each well as the percentage of respiration after the addition of each agonist respect basal respiration. Coupled respiration in each well was determined as the percentage of respiration after the stimulus (vehicle, glucagon or phenylephrine) which is sensitive to oligomycin inhibition. Maximum respiratory capacity in each well was calculated as the percentage of respiration after FCCP addition with respect to basal respiration. Non-mitochondrial
respiration (i.e. respiration that persists after treatment with rotenone and antimycin A) was subtracted from all measurements. Proton leak was calculated as the difference between oligomycin-sensitive and non-mitochondrial respiration in each well.

To obtain the effects respect to vehicle, the values of stimulation, coupled respiration, maximum respiratory capacity and proton leak of each well treated with glucagon or phenylephrine were divided by the average of vehicle treated wells in each case. The average of the four wells for each condition in each experiment was used.

3.6. Oxygen and glucose deprivation and cell death assessment by propidium iodide/calcein-AM

Primary cortical neurons after 10-11 days in vitro were subjected to oxygen and glucose deprivation (OGD). Culture medium was replaced by glucose-free Neurobasal A (NBA, Gibco), and cells were placed in an hypoxic incubator (Biospherix) at 0.6% \( O_2 \) and 37°C for 1h 30 min. In normoxic control cells, Neurobasal medium was replaced by Neurobasal A supplemented with 25 mM D-glucose, a value usually considered to be the glucose concentration found in Neurobasal (Kleman et al, 2008; Russell et al, 2002), and cells were placed in a regular incubator at 5% \( CO_2 \) for the same time length. After 1h30 minutes, NBA medium was replaced in both OGD and normoxic cells by Neurobasal supplemented with B27, Glutamax and gentamycin. Cell death was quantified after 24 hours using propidium iodide (PI)/calcein-AM staining (see below) or rinsed in RIPA buffer for western blot analysis.

Cell death was analysed in primary cortical neuronal cultures 24h after OGD by propidium iodide (PI; Molecular Probes) and calcein-AM (Molecular Probes) staining. PI binds DNA but is impermeable to the plasma membrane, therefore specifically staining dead cells, whereas native calcein is non-fluorescent and requires conversion to a fluorescent form by an intracellular esterase, staining only living cells (Galluzzi et al, 2009). The excitation/emission wavelengths of both probes (530/620 and 485/535 nm for PI and calcein, respectively) allows simultaneous staining and total live/dead cells ratios can be obtained for each field. Cells were loaded with 1 \( \mu \)M PI and 2 \( \mu \)M calcein-AM in Neurobasal and left 5-10 minutes at 37°C before taking images. 20x images from 10 fields of different wells were randomly taken using an Axioskopplus2 vertical microscope (Zeiss) coupled to a Coolspap FX CCD camera (Roper scientific) and quantification was performed using ImageJ software. Files were converted to 8-bit and a threshold was set. Images were then made binary and automatically counted. Threshold, circularity and size parameters were maintained throughout each experiment.
RESULTS
1. Primary characterization of SCaMC-3\(^{-}\) mice

1.1 Mice deficient in SCaMC-3 show no phenotypic abnormalities

The appearance of mice lacking SCaMC-3 is normal, with no evident phenotypic traits. Mice are viable and the number of embryos per pregnancy is not affected by the lack of the wild type allele (SCaMC-3\(^{+/+}\): 8 ± 0.73; SCaMC-3\(^{-/-}\): 7.3 ± 0.98) with no observed embryonic abnormalities. In order to confirm if the lack of SCaMC-3 could have some deleterious effect and to detect any sex-related bias, we designed a breeding experiment using heterozygous animals and measured the proportion of the three different genotypes (SCaMC-3\(^{+/+}\), SCaMC-3\(^{+/-}\) and SCaMC-3\(^{--}\)) in the offspring (Figure 3.1A). Three independent SCaMC-3 heterozygous males and three females were crossed with wild type mice. From each offspring of the F1, three male and three female heterozygous mice were selected. This way we obtained eighteen heterozygous mice (nine males and nine females) with the mutated allele coming from their father, and eighteen with the mutation coming from their mother. Mice from each of these groups were crossed between them in all possible combinations and the 125 mice from the F2 were analysed (Figure 3.1B). The results shows a 1:1 ratio of males and females (60 males vs 65 females), as expected. Heterozygous mice are born about two-fold compared to SCaMC-3\(^{+/-}\) and SCaMC-3\(^{+/+}\) mice, following the mendelian proportions for autosomal genes. However, when analysed by sex, a slight lower proportion of SCaMC-3\(^{+/-}\) observed in male mice. Although this difference is not significant (\(\chi^2\): 0.33; \(p\)=0.15), a small effect of SCaMC-3 deficiency on the number of SCaMC-3\(^{+/-}\) male mice born cannot be completely ruled out and might be observed using a higher sample size.

![Breeding Experiment Diagram](image)

**Figure 3.1. SCaMC-3\(^{-}\) mice are born in mendelian proportions.** A. Schematic representation of the breeding experiment. Heterozygous mice with their mutant alleles coming either from their father or their mother were crossed in all possible combinations and the F2 was analysed. B. Quantification of the genotypes obtained in F2. No significant variations from the expected mendelian proportions were observed (\(n\)=125), although a lower proportion of SCaMC-3\(^{+/-}\) male mice was observed (\(\chi^2\)=0.33, \(p\)=0.15, not significant).
1.2. Protein expression of SCaMC-3 in tissues and developmental stages

Most previous studies on the mitochondrial ATP-Mg/Pi carrier have been performed using rat liver, although the carrier activity has also been described to be present in kidney from these animals (Aprille, 1993). In humans, northern blot and real-time PCR analysis indicate a widespread expression, with SCaMC-3 mRNA being detected in pancreas, skeletal muscle, heart, lung, placenta, intestine and testis (Del Arco and Satrústegui, 2004; Fiermonte et al, 2004). To identify tissues where the absence of SCaMC-3 might be relevant in mice, discard possible dosage compensation effects of other paralogs and understand the lack of abnormalities during early development, where the carrier has been described to be important, we obtained samples from SCaMC-3<sup>−/−</sup> and wild type mice and analysed the protein levels of SCaMC-1, SCaMC-2 and SCaMC-3.

Protein expression of the three main paralogs of the ATP-Mg/Pi carrier was studied by western blot analysis in brain, liver, lung, heart, spleen, colon, kidney, duodenum and ovary from wild type and SCaMC-3<sup>−/−</sup> mice (Figure 3.2A). SCaMC-2 was only detected in brain, whereas SCaMC-1 was detected in lung, spleen, colon and ovary, and SCaMC-3 showed its highest levels in brain, liver and kidney, with low levels also observed in the rest of tissues analysed (Figure 3.2A). In SCaMC-3<sup>−/−</sup> animals, SCaMC-3 was not detected and no sign of up-regulation of the expression of other paralogs was found.

![Western Blot of SCaMC-3 in Tissues](image)

**Figure 3.2. Expression analysis of the mitochondrial ATP-Mg/Pi carrier paralogs in mouse. A.** Protein analysis by western blot of SCaMC-1, SCaMC-2 and SCaMC-3 from the tissues indicated from 3-month old wild type and SCaMC-3<sup>−/−</sup> mice using 50 μg of whole-tissue lysates. **B.** Western blot analysis of SCaMC-1, SCaMC-2 and SCaMC-3 in liver and brain lysates from embryos and pups of different developmental stages, from embryonic day 15 (E15) to post-natal day 3 (P3). The expression of Hsp60 was used as loading control. Antibodies were used at the dilution indicated in Materials and methods. Molecular weights of the bands observed are indicated.
The first paradigm where the activity of the ATP-Mg/Pi was described and the protein was proposed to play an important role was in the mitochondrial uptake of adenine nucleotides that takes place in newborn liver (Aprille and Asimakis, 1980; Sutton and Pollak, 1978), where SCaMC-3 appears to be the main form of the carrier in adults (Figure 3.2A). Yet, \( \text{SCaMC-3} \) mice do not show abnormalities on birth. Therefore, we decided to study the protein levels of the different paralogs of the ATP-Mg/Pi carrier in brain and liver, the tissues where SCaMC-3 shows higher expression, during the perinatal period, from embryonic day 15 to postnatal day 3, as well as in adult tissue. Although this approach does not allow to compare the level of the different paralogs between them, it provides the pattern of expression of each protein during late embryonic and early postnatal stages. In both brain and liver, the expression of SCaMC-3 increased progressively from late embryonic stages to adult tissue (Figure 3.2B). SCaMC-1 showed the opposite pattern in both tissues, with higher levels during late embryonic development, from embryonic days 15 (E15) to 17 (E17) and lower levels after birth, from postnatal days 0 (P0) to 3 (P3), being negligible in adult tissue (Figure 3.2B). SCaMC-2 was undetected in liver and displayed an analogous expression pattern to SCaMC-3 in brain (Figure 3.2B).

These results indicate that SCaMC-3 is the main paralog of the ATP-Mg/Pi carrier in adult liver and that is co-expressed with SCaMC-2 in adult brain. The absence of SCaMC-3 does not induce up-regulation of other paralogs in any of the tissues studied. In liver, SCaMC-1 and SCaMC-3 have an overlapping expression from embryonic day 15 (E15) until several days after birth, but not on adult tissue, where SCaMC-3 is the only detectable paralog (Figure 3.2B). The coexistence of both paralogs after birth, where the ATP-Mg/Pi carrier is thought to mediate the adenine nucleotide uptake of liver mitochondria (Aprille, 1981; Aprille, 1988; Aprille and Asimakis, 1980), could provide an explanation for the lack of defects in liver mitochondria from \( \text{SCaMC-3} \) mice.

1.3. Respiratory function in SCaMC-3-deficient mitochondria

Although oxidative phosphorylation does not require net uptake of adenine nucleotides into the mitochondria, and is maintained by the exchange of cytosolic ADP with matrix ADP by means of the ATP/ADP translocase, the absence of SCaMC-3 could have an indirect effect on respiratory rates due to alterations in the mitochondrial net adenine nucleotide content (Asimakis and Aprille, 1980a). Therefore, we studied the respiratory rates of isolated liver and brain mitochondria using a Clark electrode as indicated in Materials and methods. Figure 3.3 shows the respiratory rates and the respiratory control ratios obtained using substrates from complex I (5 mM glutamate plus 5 mM malate) or complex II (2 \( \mu \)M rotenone plus 5 mM succinate), in the absence (state 4) or presence (state 3) of 0.5 mM ADP, in liver (Figure 3.3A) and brain (Figure 3.3B) mitochondria.

In liver mitochondria (Figure 3.3A), slight differences were observed with succinate as substrate, with
SCaMC-3-deficient mitochondria displaying higher state 4 and state 3 respiratory rates, but there were no significant differences in the respiratory control ratios from SCaMC-3-deficient mitochondria. Respiration rates were higher in brain than in liver mitochondria. Brain mitochondria from SCaMC-3-deficient animals showed respiration rates similar to their wild type counterparts with all substrates (Figure 3.3B)

![Figure 3.3](image.png)

**Figure 3.3. Respiratory rates in SCaMC-3-deficient liver and brain mitochondria are similar to wild type rates.** A. Respiratory rates were measured in isolated liver (A, 0.6 mg/mL) and brain (B, 0.2 mg/mL) mitochondria from wild type (white bars) and SCaMC-3−/− (back bars) mice using substrates for complex I, 5 mM glutamate and 5 mM malate (glu+mal), or complex II, 5 mM succinate plus 2 mM rotenone, in the presence (state 3) or absence (state 4) of 0.5 mM ADP. Respiratory control ratios were calculated by dividing state 3 by state 4 respiratory rates. Results are expressed as mean ± S.E.M. of 8 (liver, complex I), 22 (liver, complex II), 7 (brain, complex I) and 11 (brain, complex II) independent experiments.

Overall, no significant differences were observed in any case, which would indicate normal electron transport chain activity and oxidative phosphorylation. In addition, citrate synthase activity, a marker of mitochondrial mass (Barrientos, 2002), did not show any difference between genotypes either in brain or liver (Table 3.1)

<table>
<thead>
<tr>
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<th>Citrate synthase activity (µmol/min/mg of protein)</th>
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<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Wild type</td>
<td>11.84 ± 2</td>
</tr>
<tr>
<td>SCaMC-3−/−</td>
<td>12.28 ± 1.48</td>
</tr>
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**Table 3.1. Citrate synthase activity in brain and liver mitochondria from wild type and SCaMC-3−/− animals.** Mitochondrial mass was evaluated using citrate synthase activity. No significant differences were observed between genotypes. Data is shown as mean ± S.E.M. (n=5 for liver mitochondria and 4 for brain mitochondria).
2. SCaMC-3 in liver: coupling between electron transport chain activity and oxidative phosphorylation

2.1. Induction of state 3 respiration and recovery of respiratory control ratios following adenine nucleotide depletion

Embryonic liver mitochondria from mammals display a low content of adenine nucleotides that increases during the first post-natal hours in a process that has been ascribed to the ATP-Mg/Pi carrier (Aprille, 1993). Adenine nucleotide uptake by mitochondria is followed by an increase in state 3 respiration (i.e. respiration dependent on ATP synthesis by the ATP synthase) and a switch from a glycolytic to an oxidative metabolism (Valcarce et al, 1988). Figures 3.5 and 3.6 show the strategy employed to deplete mitochondria from adenine nucleotides by incubation at 30°C for 5 minutes with 2 mM pyrophosphate, which is exchanged by matrix adenine nucleotides through the adenine nucleotide translocase (Asimakis and Aprille, 1980b) (see Materials and methods). Adenine nucleotide depletion can be confirmed by the inability of mitochondria to induce state 3 respiration after ADP addition in the absence of calcium, as ATP is required within the matrix to be exchanged by exogenous ADP (Asimakis and Sordahl, 1981), and therefore only net uptake of adenine nucleotides can restore state 3 respiration. Following adenine nucleotide depletion, the effect of adenine nucleotide-uptake on mitochondrial respiration was studied by two different means: 1) Monitoring state 3 respiration after addition of ADP to succinate-respiring adenine nucleotide-depleted mitochondria in the presence or absence of calcium (Figure 3.4) and 2) pre-incubation of adenine nucleotide-depleted mitochondria for different time lengths in a medium that favours ATP-Mg uptake followed by measurement of state 3 respiration with succinate in the absence of calcium (Figure 3.5).

2.1.1. SCaMC-3 mediates the induction of state 3 by ADP in adenine nucleotide-depleted mitochondria in a calcium-dependent way.

As a first means to study the effect of SCaMC-3 in coupling electron transport chain activity and oxidative phosphorylation, and as it has been described that ADP can also be transported through the ATP-Mg/Pi carrier (Fiermonte et al, 2004; Nosek et al, 1990), we measured state 3 induction by adding 2 mM ADP to adenine nucleotide-depleted liver mitochondria from wild type and SCaMC-3<sup>−/−</sup> mice, in the presence or absence of extramitochondrial calcium (Figure 3.4).

Mitochondria depleted from adenine-nucleotides were assayed in MSK medium devoid of EDTA but in the presence of 200 nM ruthenium red. As ruthenium red inhibits the mitochondrial calcium uniporter, in these conditions only extra-mitochondrial calcium is available (Hajnóczky et al, 2006). State 4 respiratory rates using 2 μM rotenone and 5 mM succinate were similar in both genotypes, but addition of 2 mM ADP induced an increase in state 3 respiration in wild type mitochondria that was not observed in SCaMC-3-deficient mitochondria (122.4 ± 18.2 vs 53.5 ± 13.7 nmol O/min/mg protein, p=0.013, unpaired, two-tailed
Figure 3.4. Schematic representation of the experiment shown in Figure 3.6. Mitochondria were depleted from adenine nucleotides and monitored for oxygen consumption. In these conditions, net uptake of ADP through calcium-activated SCaMC-3 is required to induce state 3 respiration.

Figure 3.5. Schematic representation of the experiment shown in Figure 3.7A. Mitochondria were depleted from adenine nucleotides and incubated in the presence of ATP-Mg. Uptake of ATP-Mg through calcium-activated SCaMC-3 restores matrix adenine nucleotides and allows stimulation of state 3 respiration by addition of ADP in the absence of
Student t) (Figure 3.6A). Stimulation of respiration by ADP in wild type mitochondria was dependent on ATP synthesis, as it was inhibited by addition of oligomycin, whereas uncoupled rates obtained after addition of FCCP were similar in both genotypes (Figure 3.6A). Since respiratory rates were not changed in ScAMC-3-deficient mitochondria after ADP addition, respiratory control ratios remained, as in the case of mitochondria depleted from adenine nucleotides, close to 1 (1.01 ± 0.10), whilst wild type mitochondria increased its rates ~2.5-3-fold (2.71 ± 0.44, p=0.005 unpaired, two-tailed, Student t compared to respiratory control ratios from ScAMC-3-deficient mitochondria). When the experiment was performed in MSK medium supplemented with 1 mM EGTA (i.e. in the absence of calcium) the stimulation of the respiration in wild type mitochondria after addition of 2 mM ADP could no longer be observed and respiratory rates were similar to ScAMC-3-deficient mitochondria (Figure 3.6B). These state 3 respiratory rates were undistinguishable from state 4 respiratory rates therefore rendering in both cases respiratory control ratios close to 1 (1.15 ± 0.11 in wild type mitochondria vs 1.06 ± 0.12 in ScAMC-3-deficient mitochondria).

Figure 3.6. Addition of ADP to adenine nucleotide-depleted mitochondria stimulates respiration through ScAMC-3. 0.3 mg of wild type and ScAMC-3-deficient liver mitochondria depleted from adenine nucleotides were monitored for oxygen consumption using a Clark-type electrode in the presence (A) or absence (B) of extra-mitochondrial calcium. Left part of each panel shows traces of oxygen consumption versus time from a representative experiment, whereas in the right part, state 4 rates, state 3 and uncoupled rates, as well as respiratory control ratios from 4 independent experiments are displayed. A. Adenine nucleotide-depleted wild type mitochondria respiring 5 mM succinate plus 2 µM rotenone increase respiratory rates after 2 mM ADP addition (state 3) in a medium devoid of chelating agents and with 200 nM ruthenium red (RR), which blocks calcium entry into the mitochondrial matrix through the mitochondrial calcium uniporter, whereas ScAMC-3-deficient mitochondria fail to stimulate respiration. Inhibition of respiration by oligomycin indicates that the stimulation is dependent on ATP synthesis through the ATP synthase. B. Adenine nucleotide-depleted mitochondria respiring 5 mM succinate plus 2 µM rotenone cannot induce state 3 respiration by 2 mM ADP addition when 1 mM EDTA and 1 mM EGTA are present in the medium. Results are expressed as mean ± S.E.M. of 4 independent experiments (*p<0.05; **p<0.01; wild type vs ScAMC-3−/− two-tailed, unpaired Student t; #p<0.05; ##p<0.01; state 3 vs uncoupled two-tailed, paired Student t). Scale bars: 10 nmol O (vertical), 1min (horizontal).
2.1.2 Adenine nucleotide-depleted mitochondria lacking SCaMC-3 fail to recover respiratory control ratios after incubation in a medium that favours ATP uptake.

Liver mitochondria depleted from adenine nucleotides as indicated in Materials and methods were incubated in MSK medium lacking EDTA and supplemented with 2 mM ATP, 5 mM MgCl₂ and 200 nM ruthenium red at 30°C for different time lengths. In these conditions, ATP binds to magnesium and can enter the mitochondria through the ATP-Mg/Pi carrier in exchange with matrix Pi (Aprille and Austin, 1981; Asimakis and Aprille, 1980a; Asimakis and Aprille, 1980b) (Figure 3.5). As in the previous experiment, the presence of 200 nM ruthenium red prevents cytosolic calcium from entering the mitochondria, hindering activation of calcium-dependent processes in the matrix (Hajnóczky et al, 2006). At different time points (1, 2, 3 and 10 minutes), the ATP-Mg filling reactions were stopped by introducing the samples on ice. The mitochondrial pellet was recovered by centrifugation and assayed for oxygen consumption using a Clark-type electrode in MSK medium supplemented with 1 mM EGTA (i.e. without calcium). After addition of 2 μM rotenone and 5 mM succinate, state 3 was induced by 0.5 mM ADP addition. Wild type mitochondria showed a non-significant decrease in state 4 respiration after 1 minute of incubation that was not substantially affected by longer incubation times, going from 70.94 ± 11.75 to 52.74 ± 9.07 nmol O/mg/min, whereas SCaMC-3-deficient mitochondria maintained at all incubation times values between 63.40 ± 17.05 and 67.32 ± 16.70 nmol O/mg/min (Figure 3.7A, state 4). As for state 3 respiration, wild type mitochondria increased their respiratory rates by ~50% after 2 minutes of incubation, going from 82.93 ± 15.55 to 117.56 ± 20.30 nmol O/mg/min, while the rates of SCaMC-3-deficient mitochondria did not increase significantly at any of the times of incubation, ranging values from 63.29 ± 11.31 to 78.75 ± 30.09 nmol O/mg/min (Figure 3.7A, state 3). Uncoupled rates were not affected by the incubation with ATP-Mg, although values were non-significantly higher in wild type mitochondria at all times (Figure 3.7A, uncoupled). Differences in the respiratory rates of both genotypes are highlighted when respiratory control ratios are plotted against the different incubation times, with wild type mitochondria displaying values between 2.17 ± 0.35 and 2.20 ± 0.25 nmol O/mg/min for incubation times of 2 minutes or longer, and SCaMC-3-deficient mitochondria showing values around 1 at all times measured (Figure 3.7A, RCR).

To study if the increase of adenine nucleotide content in the mitochondrial matrix could regulate oxidative phosphorylation in basal conditions (i.e. in non-depleted mitochondria), we incubated intact mitochondria in the same conditions as in the previous experiment, but with a higher ATP and MgCl₂ concentrations (10 mM each). As can be observed in Figure 3.7B, state 4 and state 3 respiratory rates were not affected in wild type or SCaMC-3-deficient mitochondria (Figure 3.7B, state 4 and state 3), despite the fact that wild type mitochondria greatly increased their adenine nucleotide content (Figure 3.7B, ATP+ADP). Interestingly, in these conditions mitochondria lacking SCaMC-3 could still modestly increase their adenine nucleotide levels, although to a substantially lower extent than wild type mitochondria (Figure
Figure 3.7. Respiration in mitochondria loaded with adenine nucleotides. A. Wild type and SCA-MC-3-deficient liver mitochondria were depleted from AdNPs and incubated at 30°C in the presence of 200 nM RR, 2 mM ATP, and 5 mM MgCl\(_2\) for different time lengths before monitoring state 4, state 3 and uncoupled respiratory rates in the presence of 2 μM rotenone plus 5 mM succinate. Respiratory control ratios (RCR) are also shown. Maximal increase of state 3 respiratory rate is observed in wild type cells after 2 min of incubation. Results are expressed as mean ± S.E.M. Of 5 independent experiments (*p<0.05; **p<0.01; two-tailed, unpaired Student t). B. Non-depleted wild type and SCA-MC-3-Δ mitochondria were incubated at 30°C in the presence of 200 nM RR, 10 mM ATP, 10 mM MgCl\(_2\) and 20 μM CaCl\(_2\) at different times before monitoring state 4, state 3 and uncoupled respiratory rates as in A. To correct for residual adenine nucleotides in the medium, state 4 was obtained after the addition of oligomycin. In parallel, mitochondrial levels of ATP + ADP were also determined (bottom panel). Results are expressed as mean ± S.E.M. of 3-5 independent experiments (*p<0.05, two-tailed, unpaired Student t).
3.7B, ATP+ADP).

Therefore, transport of ADP or ATP-Mg through SCaMC-3 promotes coupling between mitochondrial respiration and oxidative phosphorylation in conditions were mitochondria have a low adenine nucleotide content, a situation similar to the one observed in newborn liver mitochondria from mammals, but this increase in matrix adenine nucleotides does not seem to be sufficient by itself to modify the respiratory rates in intact mitochondria. As in the case of other CaMCs, this transport requires calcium, but not calcium entry in the mitochondria (Mármol et al, 2009; Palmieri et al, 2001; Traba et al, 2008).

2.2. Influence of SCaMC-3 in the effects of glucagon and phenylephrine in liver mitochondria

It has been known for a long time that glucagon and calcium-mobilising agents, such as phenylephrine, induce an increase in the content of mitochondrial adenine nucleotides through a mechanism that requires calcium, as it is inhibited by EGTA (Aprille et al, 1982; Haynes et al, 1986; Hensgens et al, 1980; Siess et al, 1977). It has been suggested that this increase could underlie the regulation of some of the ADP and ATP-dependent processes triggered by these hormones in the mitochondria (Aprille et al, 1987). In addition to increasing the adenine nucleotide content, glucagon and phenylephrine also stimulate respiration in liver mitochondria (Yamazaki, 1975; Crompton and Goldstone, 1986).

To evaluate the role of SCaMC-3 in these processes, we studied the effects of glucagon and phenylephrine in mitochondrial respiration and measured the uptake of adenine nucleotides and respiratory rates in liver mitochondria from SCaMC-3+/− mice after glucagon treatment.

2.2.1. Cytosolic calcium signals evoked by glucagon and phenylephrine are not affected by SCaMC-3 deficiency

Primary hepatocytes were obtained by perfusion with collagenase and separation of cells by isodensity centrifugation in Percoll, as indicated in Materials and methods. SCaMC-3 expression in the wild type hepatocytes was confirmed by western blot of cell lysates, whereas SCaMC-1 could not be detected (Figure 3.8A). The expression of SCaMC-2 was not analysed, as we have always failed to detect this paralog in liver (Figure 3.2). To rule out possible alterations in the cytosolic calcium signals produced by glucagon and phenylephrine addition on SCaMC-3-deficient hepatocytes, cytosolic calcium levels were measured in primary hepatocytes loaded with the probe Fura-2-AM. Prior to addition of substrates, some cells showed a regular calcium-oscillatory pattern (not shown). Addition of glucagon produced asynchronous cytosolic calcium peaks of about 700 nM in most cells with a delayed onset of about 2 minutes (Figure 3.8B), and an increase in the oscillatory pattern in previously oscillating cells (not shown). As for phenylephrine, addition of the agonist induced an immediate increase in cytosolic calcium, which reached concentrations of ~500 nM (Figure 3.8C) and like in the case of glucagon, cells that showed oscillatory behaviour before the addition increased the frequency of the peaks (not shown). No differences on the amplitude or frequency of the peaks was observed between genotypes.
Figure 3.8. Cytosolic calcium signals evoked by glucagon and phenylephrine are not affected by the absence of SCaMC-3. A. Western blot from primary hepatocytes to determine the presence of SCaMC-3 and SCaMC-1. Antibodies were used at the dilutions indicated in Materials and Methods. Expression of β-ATPase was used as loading control. (B) and (C) show representative traces (upper panels) and ratio images (lower panels) of Fura-2-AM-loaded hepatocytes before and after addition of glucagon (B) or phenylephrine (C) obtained using the Aquacosmos software (see Materials and methods). Fluorescence was measured at 510 nm using two alternate excitation wavelengths, 340 and 380 nm, that specifically excitate calcium-bound and calcium-free Fura-2-AM respectively. Lower panels are representative experiments (not correspondent to the traces in the upper panel) where ratio images of both wavelengths are shown in pseudocolour, with colours ranging from blue as the lowest ratio to red as the highest. Time on each image indicates time lapse after agonist addition. Arrows point to cells with a cytosolic calcium peak and illustrate the differences in the simultaneity of the signals between both agonists. Addition of 0.1 μM glucagon induced a delayed calcium response which was indistinguishable between wild type and SCaMC-3-deficient cells (B, upper panel), whereas addition of 100 μM phenylephrine produced a peak of similar amplitude in both genotypes (C, upper panel). Scale bars: 10 μm; wt: wild type.

2.2.2. Glucagon and phenylephrine exert effects on mitochondrial respiration through SCaMC-3

Calcium signals increase mitochondrial respiratory rates by several pathways (see Introduction). Among them, mitochondrial adenine nucleotide uptake through the calcium-dependent ATP-Mg/Pi carrier activity may play a significant role in glucagon and calcium-mobilising agents signalling in liver (Aprille, 1993;
Figure 3.9. Effects of glucagon and phenylephrine on respiratory parameters in primary hepatocytes from wild type and SCaMC-3<sup>−/−</sup> animals. A, The left panel shows a representative experiment of OCR in primary hepatocytes from wild type and SCaMC-3<sup>−/−</sup> animals and the response to phenylephrine is shown. OCR is expressed as the rate at each point with respect to the basal rate at the time of addition of the agonist. Where indicated, 100 μM phenylephrine, 6 μM oligomycin, 1 μM FCCP and 1 μM rotenone plus 1 μM antimycin A (rot + AntA) were injected. On the right panel, OCR responses to phenylephrine (100 μM) and glucagon (0.1 μM) and corresponding vehicle in hepatocytes from wt and SCaMC-3<sup>−/−</sup> mice are represented. Data correspond to representative experiments. B, Respiratory parameters after treatment with 0.1 μM glucagon or 100 μM phenylephrine with respect to vehicle in wild type and SCaMC-3-deficient hepatocytes. Non-mitochondrial respiration (the lowest value remaining after rotenone plus antimycin A addition) was subtracted from all measurements after confirming that at longer times respiration was not further decreased. Respiratory parameters were calculated as the average of three consecutive measurements after each addition (agonist, oligomycin, FCCP). The stimulations of glucagon and phenylephrine and maximal respiratory capacity were calculated as the percentage of respiration after agonist or FCCP addition with respect to basal respiration. Coupled respiration is the percentage of respiration in the presence of agonist sensitive to oligomycin inhibition. Proton leak was calculated as the difference between oligomycin-sensitive and non-mitochondrial respiration. Results are expressed as mean ± S.E.M. of 3 independent experiments with four replicates each (*p<0.05; **p<0.01; unpaired, two-tailed Student t, WT vs KO; # p<0.01 ## p<0.001 two-tailed Student t, WT vs vehicle).

Titheradge and Haynes, 1980). To examine this possibility, we evaluated mitochondrial respiratory activity after glucagon or phenylephrine addition using a Seahorse XF 24-3 Analyzer, which allows for oxygen consumption measurements using intact, attached cells.

Figure 3.9A shows a representative oxygen consumption measurement experiment using the Seahorse Analyzer, indicating the time of addition of the different agonists. Addition of 0.1 μM glucagon or 100 μM phenylephrine increased respiratory rates about 20% in wild type cells (119.4 ± 0.5% and 119 ± 3%, respectively), compared to only ~5% in SCaMC-3-deficient cells (105.3 ± 2.4% for glucagon and 103 ±
5% for phenylephrine) respect to vehicle (Figure 3.9B, agonist-induced stimulation). In the case of wild type cells, most of this increase correlates with a concomitant increase in coupled respiration (i.e. oligomycin sensitive), specially in the case of glucagon (113.2 ± 2.6%, compared to 107.4 ± 3.5% for phenylephrine) (Figure 3.9B, coupled respiration). Conversely, SCaMC-3-deficient cells did not increase their coupled respiratory rates, in the case of glucagon (100.1 ± 1.4%), and even slightly decrease it with phenylephrine (91 ± 4%, not significant) (Figure 3.9B, coupled respiration). Maximum respiratory rates after dissipation of the mitochondrial proton gradient were increased in wild type cells treated with glucagon (123.3 ± 4.4%) but remained unchanged in SCaMC-3-deficient hepatocytes (102.6 ± 3.4%), whereas in the case of phenylephrine maximum rates increased to a lesser extent in wild type hepatocytes (106.5 ± 7.9%) and modestly decreased it in SCaMC-3-deficient cells (84.3 ± 9.8%) (Figure 3.9B, maximal respiratory capacity).

These results indicate that SCaMC-3 mediates the stimulation in respiration observed in hepatocytes after glucagon and phenylephrine treatment. In the case of glucagon, this stimulation correlates with an increase in the rates of coupled respiration and maximal respiratory rates and with a decrease in proton leak, all of which are absent in SCaMC-3-deficient hepatocytes (Figures 3.9B). Phenylephrine affected these three parameters in a similar way in wild type hepatocytes, although the effect was milder, and appeared to be relatively toxic in SCaMC-3-deficient cells, decreasing coupled respiration and maximum respiratory capacity, and increasing proton leak (Figures 3.9B). The reason for this is unclear, as calcium signals evoked by the two agonists appear to be within the same range (see above). However, we and others (Crompton and Goldstone, 1986) have observed that, after stimulation with phenylephrine, cytosolic calcium levels in hepatocytes do not return to baseline, but rather remain higher than before stimulation. As discussed below, it is possible that phenylephrine calcium signals cause a heavier burden on SCaMC-3-deficient mitochondria, as a consequence of reduced CRC (Traba et al, 2012 and see below).

2.2.3. The increase in liver mitochondrial adenine nucleotide content and respiratory rates after intraperitoneal administration of glucagon does not occur SCaMC-3−/− mice

To assess the response to glucagon treatment in vivo, we administered glucagon (2 mg/kg) intraperitoneally to 3-month old male wild type and SCaMC-3−/− mice (n=5-6) fed ad libitum and sacrificed them by cervical dislocation after 15 minutes. Oxygen consumption was monitored in liver mitochondria from these animals using 5 mM glutamate plus malate, 2 μM rotenone plus 5 mM succinate or TMPD plus ascorbate as substrates. Figure 3.10A shows that wild type mitochondria respond to glucagon treatment by increasing all their respiratory rates (state 4, state 3 and uncoupled) when using substrates from complex I and II, but not TMPD plus ascorbate, as has been previously described (Yamazaki, 1975; Hales trap, 1987). On the other hand, glucagon-treated mitochondria lacking SCaMC-3 displayed similar respiratory rates as vehicle-treated mitochondria.
Figure 3.10. SCaMC-3 mediates glucagon-stimulated mitochondrial respiratory activity and uptake of adenine nucleotides in vivo. A. Following administration of glucagon (2 mg/kg) or vehicle, liver mitochondria from wild type and SCaMC-3−/− mice were rapidly isolated and assayed for respiration with different electron transport chain substrates. Results are expressed as mean ± S.E.M of 6 independent experiments. (*p<0.05; two-tailed, paired Student t; **p<0.01; two-tailed, paired Student t). B and C. Mitochondrial adenine nucleotide content measured by HPLC after glucagon or vehicle treatment. Results are expressed as mean ± S.E.M of 3-5 independent experiments. (*p<0.05; wild type vs SCaMC-3−/−, two-tailed, unpaired Student t; #p<0.05; wild type vehicle vs wild type glucagon, one-tailed, unpaired Student t).

In addition to respiratory rates, the mitochondrial adenine nucleotide content was measured in glucagon-treated animals. In this case, livers were rapidly excised after killing the mice, mitochondria were extracted using a fast isolation protocol (see Materials and methods) and the adenine nucleotide content of these mitochondria was measured by HPLC. Figure 3.10B shows how mitochondria from wild type livers substantially increased their adenine nucleotide content after glucagon treatment compared to mitochondria from vehicle-treated mice, going from 12.7 ± 2.7 nmol/mg of protein in vehicle-treated mice to 19.8 ± 2.4 nmol/mg of protein in glucagon-treated mice, consistent with previous observations (Aprille et al, 1982; Hensgens et al, 1980). However, mice lacking SCaMC-3 failed to increase their adenine nucleotide content in response to glucagon and showed similar values to wild type vehicle-treated mitochondria regardless of the treatment (9.8 ± 2.6 vs 9.5 ± 2.8 nmol/mg of protein respectively) and were
significantly lower compared to glucagon-treated wild type mice (p=0.016, unpaired, two-tailed Student t) (Figure 3.10B). Interestingly, the increase in adenine nucleotide levels observed in wild type mitochondria after glucagon treatment was not restricted to a particular type of adenine nucleotide, but rather to all three forms (Figure 3.10C), which is consistent with the view supported by Aprille, by which transport through the ATP-Mg/Pi carrier does not affect the proportion between different adenine nucleotides within the mitochondria, but rather their total content, as the transport reaction is slow and transported adenine nucleotides equilibrate into all three forms by means of other reactions (Aprille, 1988). It is also interesting to note that SCaMC-3-deficient mitochondria seem to have lower levels of adenine nucleotides (with significant differences in the case of ATP) even in vehicle-treated conditions (0.84 ± 0.19 vs 1.31 ± 0.08 nmol ATP/mg of protein), which could indicate a reduction on mitochondrial adenine nucleotides in vivo in basal conditions (Figure 3.10C).

2.2.4. Liver mitochondria from SCaMC-3-deficient mitochondria show reduced CRC after glucagon treatment

Mitochondria from glucagon-treated animals show increased CRC (Prpić et al, 1978; Yamazaki, 1975). It has been described that mitochondrial calcium accumulation is highly dependent on matrix adenine nucleotides (Carafoli et al, 1965) and that SCaMC-1 deficiency in cancer lines reduces CRC in an adenine nucleotide-dependent way (Traba et al, 2012). Previous unpublished experiments from our laboratory indicate that liver mitochondria from SCaMC-3−/− mice display low CRC in the presence of 1 mM ATP-Mg or ADP, most likely due to the inability of these mitochondria to uptake adenine nucleotides required for efficiently buffer calcium (Javier Traba, PhD thesis). Since we have shown that glucagon treatment fails to increase the matrix adenine nucleotide content in the absence of SCaMC-3, we studied if CRC is also impaired in these mitochondria. Figure 3.11A shows that in the absence of external adenine nucleotides, lack of SCaMC-3 does not affect CRC. When 0.2 mM ADP (or 0.2 mM ATP-Mg, not shown) is present in the medium, CRC is greatly increased in both genotypes to a similar extent, probably by a mechanism mediated by the ANT (Kokoszka et al, 2004) (Figure 3.11B). However, when 1 mM ATP-Mg (or ADP, not shown) is present in the medium, SCaMC-3-deficient mitochondria show similar CRC values, whereas wild type mitochondria greatly increase their CRC to around 600 nmol Ca2+/mg protein (Figure 3.11C). These results indicate that transport of adenine nucleotides into the matrix through SCaMC-3 increases the CRC by a mechanism independent of the ANT. Since liver mitochondrial adenine nucleotide uptake in response to glucagon is impaired in SCaMC-3−/− animals (Figure 3.10B), we studied whether the CRC of liver mitochondria from these animals is also affected. Glucagon treatment induced an increase in CRC in the absence of external adenine nucleotides in mitochondria from wild type mice that did not take place in their SCaMC-3-deficient counterparts (Figure 3.11D), the difference between both genotypes being highly significant (244.2 ± 35.8 vs 111.7 ± 5.9 nmol Ca2+/mg protein, p=0.005, unpaired two-tailed student t).
Figure 3.11. SCaMC-3 mediates glucagon-stimulated increase in calcium retention capacity in liver mitochondria. Mitochondrial Ca\(^{2+}\) uptake was monitored using the fluorescent indicator Calcium Green 5N in the absence of adenine nucleotides in the medium (A) or in the presence of 0.2 mM ADP (B) or 1 mM ATP-Mg (C). Arrows indicate additions of 20 nmol CaCl\(_2\). Quantification of total calcium retained by mitochondria in each case is shown on the right side of each panel. Results are expressed as mean ± S.E.M. of 3-5 independent experiments. (*p<0.05; unpaired, two-tailed Student t; **p<0.01; unpaired, two-tailed Student t). Results from panels A-C are from Javier Traba’s PhD thesis (2010).

These results indicate that the absence of SCaMC-3 affects the uptake of adenine nucleotides and the increase in CRC and respiration observed after glucagon treatment, suggesting an important role of the protein in the transduction of glucagon signals into liver mitochondria.

2.3. SCaMC-3\(^{-/-}\) mice show reduced urea levels

Glucagon secreted by α-pancreatic cells from the islets of Langerhans during fasting stimulates hepatic gluconeogenesis and ureogenesis. We have shown that glucagon also induces mitochondrial adenine nucleotide uptake in liver, stimulation of respiration and enhancement of mitochondrial CRC through a pathway that requires SCaMC-3. A causal link between the uptake of adenine nucleotides by the liver mitochondria and the stimulation of gluconeogenesis and urea synthesis has been suggested, as both pathways have ATP-dependent matrix steps (Aprille, 1993; Aprille, 1988). In the case of gluconeogenesis, the enzyme pyruvate carboxylase catalyses the conversion of pyruvate into oxaloacetate, a reaction that, in some conditions, is the rate-limiting step of the pathway (Aprille et al, 1987; Aprille et al, 1981; Brennan and Aprille, 1985). As for the urea cycle, two molecules of mitochondrial ATP are required for the synthesis of carbamoyl phosphate from HCO\(_3\) and NH\(_3\) by carbamoyl phosphate synthetase (Goldstein and Aprille, 1982). Therefore, to study the if SCaMC-3\(^{-/-}\) mice are able to fully induce gluconeogenesis and urea synthesis, we subjected the animals to a 24-hour period of fasting followed by
Figure 3.12. Changes in glucose and urea blood levels after fasting and feeding. Wild type and SCaMC-3-/- 3-month old mice were subjected either to a 24h fasting followed by feeding or a prolonged 48h fasting. Blood glucose (A) and urea (B) levels were examined in the situations indicated. Glucose and urea were measured using commercial kits, as indicated in Materials and methods. Data are presented as mean ± S.E.M. (*p<0.05, unpaired two-tailed Student t; basal values of glucose and urea from the 24h fasting and feeding and the 48h fasting groups were pooled together in A and B, with n=15 for basal glucose and n=9 for basal urea levels; the rest of values in A and B were obtained with n=4-5, except glucose levels after 48h fasting, where n=10; rates from C and D were calculated for each mouse with n=4-5, except for glucose in the basal-48h fasting interval, where n=9-10).

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<th>Biochemistry</th>
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<tr>
<td>Glucose (mg/dL)</td>
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<td>182.33 ± 7.82</td>
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<tr>
<td>Urea (mg/dL)</td>
<td>44.33 ± 1.78</td>
<td>40.33 ± 4.26</td>
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<td>Triglycerides (mg/dL)</td>
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<td>154.00 ± 36.95</td>
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<td>Total cholesterol (mg/dL)</td>
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<td>101.33 ± 33.95</td>
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<tr>
<td>Alanine transaminase; ALT (=glutamic pyruvic transaminase; GPT) (U/L)</td>
<td>170.67 ± 6.34</td>
<td>240.67 ± 27.9*</td>
</tr>
<tr>
<td>Aspartate transaminase; AST (=glutamic oxaloacetic transaminase; GOT) (U/L)</td>
<td>322.00 ± 93.85</td>
<td>481.33 ± 55.81</td>
</tr>
<tr>
<td>Gamma-glutamyl transpeptidase; GGT (U/L)</td>
<td>1.67 ± 0.41</td>
<td>4.00 ± 0.71*</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.62 ± 0.08</td>
<td>0.64 ± 0.13</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dL)</td>
<td>0.29 ± 0.05</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>112.00 ± 30.21</td>
<td>138.67 ± 26.88</td>
</tr>
<tr>
<td>Total serum protein (g/dL)</td>
<td>6.80 ± 0.82</td>
<td>6.67 ± 1.02</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>5.53 ± 0.89</td>
<td>6.27 ± 0.45</td>
</tr>
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</table>

Table 3.2. Serum levels of different markers in wild type and SCaMC-3-/- mice. Serum from wild type and SCaMC-3-/- was analysed for a number of different physiological markers. A significant increase in liver enzymes was observed in SCaMC-3-/- samples, suggesting liver injury. Data shown as mean ± S.E.M. (n=3) (*p<0.05, two-tailed, unpaired Student t).

free access to food during another 24 hours, or to a 48-hour period of fasting, and measured glucose and urea levels (Figure 3.12).

No differences in blood glucose levels between wild type and SCaMC-3-/- mice were observed under basal conditions or during the fasting-refeeding protocol. However SCaMC-3-/- mice maintained higher levels of blood glucose after a 48-hour fasting period (137.1 ± 8.4 vs 115.8 ± 6.5 mg/dL; p=0.049, unpaired, two-tailed Student t, n=10) (Figure 3.12A).

On the other hand, urea plasma levels were diminished in SCaMC-3-/- mice (Figure 3.12B), with a
reduction of around 20% compared to their wild type counterparts (24.58 ± 2.12 vs 31.65 ± 2.14 mg/dL; p=0.02, unpaired, two-tailed Student t, n=10). However, no other changes in urea levels were observed during the fasting-refeeding protocol. The decrease in plasma urea levels under basal conditions suggests that ureogenesis might be affected in SCaMC-3-deficient mice, perhaps due to the failure to accumulate adenine nucleotides in liver mitochondria.

2.4. Deficiency of SCaMC-3 increases plasma levels of liver injury markers

Plasma from wild type and SCaMC-3−/− animals were analysed for a wide variety of markers that are summarised in Table 3.2. Lipid metabolism does not seem to be affected, with normal triglycerides and cholesterol levels and no excess of alkaline phosphatase or bilirubin in plasma. As for kidney markers, where SCaMC-3 expression is relatively high, creatinine and uric acid are not elevated, suggesting normal renal function. However, differences were observed in the level of liver enzymes. Alanine transaminase (ALT) levels are increased in SCaMC-3−/− mice about ~40%, whereas gamma-glutamyl transpeptidase (GGT) levels are more than two-fold higher than in wild type mice. SCaMC-3-deficient mice also show high levels of aspartate transaminase (AST), although differences with wild type mice were not significant. These observations suggest some type of liver injury in SCaMC-3-deficient mice.

3. SCaMC-3 in brain: susceptibility to calcium-induced cell damage

3.1. Expression pattern of SCaMC-3 in brain tissues and neuronal cell cultures

In addition to liver, SCaMC-3 is also expressed in brain, where is co-expressed with the SCaMC-2 paralog (Figure 3.2A). In order to further characterise the expression pattern in brain areas and to study the cellular distribution of the paralogs in different cerebral cell types, we used western blot analysis in brain dissected areas and analysed protein expression in primary cultures and brain sections using immunocytochemistry, β-galactosidase detection and western blot analysis.

SCaMC-3−/− mice were generated by introducing the lacZ gene in the SCaMC-3 locus (see Materials and methods). Therefore, the expression of β-galactosidase in tissues from SCaMC-3−/− mice is regulated by the same promoter as SCaMC-3 in wild type mice (Contreras et al, 2010b). Due to the inability of the SCaMC-3 antibodies to recognize the protein by immunocytochemistry, and in order to study the pattern of expression of SCaMC-3 in different brain regions, we obtained brain slices from SCaMC-3−/− mice and used the X-Gal reaction to detect β-galactosidase expression. Figure 3.13 shows four different representative images. The strongest staining is seen in anterior olfactory nuclei from the olfactory bulb (Figure 3.13A), in the caudoputamen region of the striatum (Figure 3.13B), in the CA1 and dentate gyrus areas of the hippocampus (Figure 3.13C) and in the Purkinje layer of the cerebellum (Figure 3.13D). Lower signals are also observed in different layers of the cortex and in hippocampal areas CA2 and CA3, as well as the piriform cortex. No signal was observed in wild type brain sections (not shown). A semiquantitative
Figure 3.13. Representative images of brain sections from \textit{SCaMC-3}−/− animals after β-galactosidase detection using X-Gal. 40 μm brain sections after incubation with X-Gal reactive are shown with its correspondent reference area as represented in the Allen Mouse Brain Atlas (Lein et al, 2007). A being the most anterior and D the most posterior. Lower panels represent magnifications of squared areas A. Section comprising olfactory areas and isocortex. Staining can be detected in the anterior olfactory nuclei (lower panel) and cortical layer 2/3 (arrows). B. Section including cerebral cortex, cerebral nuclei (striatum and pallidum) and anterior brainstem. X-Gal signal is detected in the caudoputamen (area enclosed by discontinuous dots), cortex layer 6b (lower right panel), cortex layer 2/3 (arrows) and piriform area (lower left panel). C. Section showing cerebral cortex, hippocampus, cerebral nuclei and brainstem. X-Gal staining is high in the hippocampus (lower panel), mainly in the dentate gyrus (DG; granule cell and polymorph layers), and CA1 area. D. Section including cerebellum and posterior brainstem. Lower panel is a magnification of the cerebellar cortex, where staining is observed in the Purkinje layer. Reference images and names of the rest of the areas can be found at the Allen Mouse Brain Atlas website (http://mouse.brain-map.org). Scale bars: 0.5 mm.
<table>
<thead>
<tr>
<th>Region</th>
<th>Score</th>
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<tbody>
<tr>
<td>Hippocampus CA1</td>
<td>+++</td>
</tr>
<tr>
<td>Hippocampus CA2</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus CA3</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus dentate gyrus</td>
<td>+++</td>
</tr>
<tr>
<td>Piriform Cortex</td>
<td>+</td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
</tr>
<tr>
<td>Caudata Putamen</td>
<td>++</td>
</tr>
<tr>
<td>Anterior olfactory nuclei</td>
<td>+++</td>
</tr>
<tr>
<td>Purkinje cell layer (cerebellum)</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 3.3. Quantification of X-Gal signal in brain slices from SCA MC-3-/- animals. X-gal staining from images from Figure 4.11 was evaluated using a semi-quantitative method, from + (low signal) to +++ (high signal).

quantification by regions is shown in Table 3.3.

Although β-galactosidase detection in brain sections appears to be restricted to certain brain areas, attention must be called to the fact that this protein is cytosolic, whereas SCA MC-3 is embedded in the inner mitochondrial membrane. Although in astrocytes this fact is of less importance, in neurons most mitochondria seem to originate in the soma and are transported through the axon to areas with high energetic demands, which can be at great distance (Sheng and Cai, 2012). Therefore, the X-Gal reaction provides information about where is the protein synthesised, but not where it is located and exerts its function. To assess this issue, and to analyse the expression of SCA MC-2, since our antibody does not recognize the protein in immunohistochemistry, we dissected brain areas and studied the protein levels of SCA MC-3 and SCA MC-2 by western blot (Figure 3.14A). Interestingly, this analysis indicates that both proteins are found at similar levels throughout all brain areas, with no differences in any particular region.

To discriminate whether SCA MC-3 and SCA MC-2 are expressed in neurons and astrocytes we performed primary cultures of cortical neurons and astrocytes from SCA MC-3-/- animals and analysed the expression of the SCA MC-3 locus using the X-Gal reaction combined with immunocytochemistry with antibodies for markers specific for neurons (microtubule associated protein; MAP2) and astrocytes (glial fibrillary acidic protein; GFAP) (Figura 3.14B). SCA MC-3 protein was detected in both types of cultures, neuronal and astrocytic confirming protein expression in both cell types (Figura 3.14C). SCA MC-2 was also only observed in neuronal cultures (Figura 3.14C).

3.2. Adenine nucleotide levels in brain mitochondria from SCA MC-3-/- mice are not significantly decreased

Data from liver mitochondria, where mitochondrial ATP is significantly decreased in basal conditions (Figure 3.10C), prompted us to analyse the levels of adenine nucleotides in brain mitochondria. We extracted brain mitochondria from wild type and SCA MC-3-/- mice and measured the levels of adenine
Figure 3.14. SCaMC-3 expression in brain areas and primary cortical cultures of neurons and astrocytes. A. Western blot using 30 μg of total protein from dissected brain areas from wild type mice. Levels of β-ATPase were determined as loading control. All antibodies were used at the dilutions indicated in Materials and methods. Regions are abbreviated as follows: o.b.: olfactory bulb; cbl: cerebellum; hip: hippocampus; ctx: cortex; str: striatum; s.c.: spinal chord. B. Representative images from neuronal and astrocyte cultures from SCaMC-3^{−/−} mice indicating co-localization of β-Gal and the fluorescent signals from neuronal marker NeuN and astrocyte marker GFAP, indicating that the locus is active in both cell types. C. Western blot analysis using 10 μg of lysates from cortical neurons and astrocytes cultures. MAP2 and GFAP were used as markers of neurons and astrocytes, respectively. Hsp60 was used as loading control.

nucleotides by HPLC (Figure 3.15). As in the case of liver, the overall levels of adenine nucleotides were lower in SCaMC-3-deficient brain mitochondria compared to wild type mitochondria (10.3 ± 1.7 vs 12.98 ± 2.52 nmol/mg of protein) but this difference was not statistically significant (p=0.35, unpaired two-tailed Student t) (Figure 3.15A). The levels of the individual nucleotides (AMP, ADP and ATP), as in the case of liver, were also reduced in all cases in SCaMC-3-deficient brain mitochondria, with the highest differences observed for ADP, but were not significantly different (2.75 ± 0.42 vs 3.7 ± 0.6 nmol/mg of protein; p=0.19 unpaired, two-tailed Student t) (Figure 3.15B).

3.3. Intraperitoneal administration of kainic acid induces a more severe seizure phenotype and a larger inflammatory response in mice deficient in SCaMC-3.

It has been described that overexpression of SCaMC-1, the mitochondrial ATP-Mg/Pi carrier present in tumour cells, reduces susceptibility against mitochondrial calcium overload and provides resistance to calcium-mediated cell death (Traba et al, 2012). Conversely, unpublished results from our laboratory using brain and liver mitochondria from SCaMC-3^{−/−} mice indicate that the lack of SCaMC-3 reduces CRC in the presence of adenine nucleotides and that primary cortical neurons from SCaMC-3^{−/−} mice are more susceptible to glutamate-induced cell death, which has been proposed to be mediated by mitochondrial calcium overload (Li et al, 2009).

Glutamate is the major excitatory neurotransmitter in the brain and can act on both ionotropic and
metabotropic receptors (Bloss and Hunter, 2010). Among the first, binding to N-methyl-D-aspartate (NMDA) receptors induces calcium entry in the cytoplasm which can be toxic if activation is excessively maintained (Bowie, 2008). In addition, other types of glutamate ionotropic receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainic (2-(2-carboxy-4-isopropenyl)pyrrolidin-3-yl)acetic acid) acid (KA) receptors are also permeable to calcium and have been shown to mediate some of the neurotoxic effects of glutamate (Cheng and Sun, 1994). KA is a natural analogue of glutamate about 30-fold more active (Bleakman and Lodge, 1998) which is used as a model for temporary lobe epilepsy (Gröticke et al, 2008). Its administration induces seizures that originate in CA3 area in the hippocampus and neuronal cell death in discrete areas that are reminiscent of the human pathology (Ben-Ari, 1985). KA displays high affinity for KA receptors (Kd of about 5-100 nM) and much lesser for AMPA receptors (Kd in the μM range) (Vincent and Mulle, 2009). However, KA receptors show fast agonist-induced desensitization, which suggest that a significant part of KA effect is mediated through AMPA receptors (Vincent and Mulle, 2009). Neuronal damage after KA administration is mainly located in the regions CA1 and CA3 of the hippocampus, where a higher density of KA receptors exists (Darstein et al, 2003).

In order to analyse the increased susceptibility of SCaMC-3-deficient neurons to glutamate-induced cell death in an in vivo context, we studied excitotoxicity by injecting KA intraperitoneally to SCaMC-3+/− and wild type mice and examined the effect on seizures, cell death and inflammatory response.

After KA administration, the appearance of seizures was monitored visually for almost two hours using a scoring scale previously adapted from the Racine's scale (McLin and Steward, 2006). This scale goes from 1 (no effect) to 7 (death), where levels 2 and 3 are pre-seizure levels, level 4 refers to a strong
Figure 3.16. *SCaMC-3−/−* mice show increased susceptibility to KA-induced seizures. Mice were administered intraperitoneally with KA (25 mg/kg) and seizure appearance and severity were monitored using Racine's scale (see Materials and methods). A. Seizure profile of wild type and *SCaMC-3−/−* mice after KA treatment. Seizure appearance, with the most severe state every 5 minutes, is plotted against time for 2 hours. For the statistical analysis, normality and variance homogeneity of the data were confirmed using Shapiro-Wilk's and Levene's tests, respectively, and a factorial ANOVA was performed using the severity of the crisis as the dependent variable and time and genotype as independent variables (n=8 male mice from each genotype, *p<0.05). B. Mean values of different parameters of seizure appearance and severity: "latency time" is the time of appearance of the first state 4 crisis after KA administration; "developed crisis" is the number of state 4 and state 5 crisis developed during the experiment; "accumulative score" is the result of arithmetically adding all states recorded and "mean level" is the mean of those states (*p<0.05; ***p<0.001 paired, two-tailed Student t).

Seizure episode and level 5 to continuous seizures (see Materials and methods). Since level 6 is usually precursor to death, we tested different KA doses and chose 25 mg/kg, whose effects ranged between levels 3 to 5 (not shown). Mice were paired by age (3 month-old, most of them littermates) and by weight (average weights were 32.88 ± 1.22 g for wild type mice and 32.77 ± 0.79 g for *SCaMC-3−/−* mice). Eight male mice from each genotype were used.

Figure 3.16A shows the seizure profile of animals from both genotypes during two hours after KA administration. Mice lacking *SCaMC-3* display a worse outcome which is already evident after 10 minutes and that is maintained throughout the experiment. All parameters calculated to evaluate the appearance and severity of seizures are consistent with an increased susceptibility in *SCaMC-3−/−* animals, with mice showing a higher number of crisis that are triggered earlier (Figure 3.16B). Deficiency in *SCaMC-3* reduced the latency time (i.e. time of appearance of the first state 4 crisis) from 80 ± 13 to 24 ± 2 minutes (p=0.0002 paired two-tailed Student t) and increased the total number of developed crisis (i.e. number of
state 4 and 5) which was 0.75 ± 0.86 in wild type mice and 2.75 ± 1.25 in SCaMC-3/− mice (p=0.03 paired, two-tailed Student t). The mean level (i.e. mean seizure state of each mouse) was highly increased in SCaMC-3/− mice (2.61 ± 0.05, compared to 1.68 ± 0.07 in wild type mice; p=6 x 10^{-10}), and the accumulative score (i.e. mean value for the sum of states throughout the experiment for each mouse) was also higher in SCaMC-3/− mice (35.25 ± 6.39 in wild type mice vs 54.87 ± 6.09 in SCaMC-3/− mice; p=0.02 paired, two-tailed Student t). These results indicate that SCaMC-3/− mice are more susceptible to KA-induced seizures than their wild type littermates.

Excitotoxicity by KA induces astroglia, which is considered a marker of neurotoxicity and strong neuronal activity (Chen et al, 2005). It is characterised by an increase in the number and volume of astrocytes and the activation of microglia in the hippocampus. Both cell types regulate the inflammatory response by releasing cytokines, though it is not clear whether they exert a neuroprotective role or contribute to cell damage (Zhang and Zhu, 2011). Seven days after KA administration, animals were sacrificed and brain sections obtained. Astroglia in the hippocampus was evaluated by immunohistochemistry against GFAP, a glial marker. Figure 3.17A shows representative images of wild type and SCaMC-3/− mice, with a more intense signal being detected in the latter (27.52 ± 4.33% vs 15.05 ± 3.94% of area occupied by GFAP fluorescence) (quantified in Figure 3.17B), indicating a higher astroglia response.

To analyse cell death, Fluoro-Jade B staining, which specifically marks degenerating or damaged neurons (Schmued et al, 1997), was used in brain sections. Surprisingly, despite differences observed in seizures and inflammation between genotypes, positive staining was only detected in two mice of each genotype and was only loosely correlated with seizure severity (Table 3.4). The mice most affected by seizures from each genotype that could be analysed, the wild type mouse from pair #3 (Figure 3.17C, left panel) and the SCaMC-3/− mouse from pair #1 (Figure 3.17C, right panels), showed positive staining for Fluoro-Jade B in the CA3 region, which was stronger in the case of the wild type mouse. The SCaMC-3/− mouse with the most severe seizure phenotype (the mouse from pair #2) died six days after KA treatment and its brain could not be extracted for histological analysis. Yet, Fluoro-Jade B staining was not observed in the SCaMC-3/− mouse from pair #3, which had a strong seizure phenotype. However, it was observed (although at very low levels) in the CA1 area from the wild type mouse from pair #5 (not shown), which did not reach state 4 at any time (Table 3.4). H&E staining in brains from these animals was similar in both genotypes (Figure 3.17D) and no differences in cell number (not shown) or intensity of the stain were observed. Higher doses of KA are probably required to better observe hippocampal damage, as it has been described that mice with this genetic background (C57BL6/Sv129) are more resistant than other strains towards neuronal damage (McKhann et al, 2003) (see Discussion).
Figure 3.17. Histological analysis of mouse brains following KA administration. A. Immunohistochemistry of representative hippocampal sections from KA-treated wild type and SCA3C-3-deficient brains using GFAP antibody, and detail from CA1 area. B. Fluorescence quantification of GFAP in hippocampal areas represented as the percentage of hippocampus area occupied by GFAP signal using ImageJ software (see Materials and methods) (n=7; **p<0.01 paired, two-tailed Student t) Images were taken and processed using identical conditions and settings. C. Neuronal damage observed with Fluoro-Jade B in CA3 regions (arrows) in brains from KA-treated wild type mouse #3 (left panel) and SCA3C-3C mouse #1 (right panels). D. H&E staining in brain sections from KA-treated wild type and SCA3C-3C mice. Staining of hippocampal cells was similar in both genotypes, indicating no differences in cell death. Scale bars: 400 μm.

### 3.4. Mice lacking SCA3C-3 are protected against ischemia/reperfusion injury

As a second model to study calcium-induced cell death in brain, we studied the effect of the absence of SCA3C-3 in ischemia/reperfusion, a paradigm where calcium-overload has been described as a major cause of neuronal death (Hertz, 2008; Sims and Muyderman, 2010). Two approaches were performed: in the first one, mice were subjected to middle cerebral arterial occlusion followed by reperfusion and infarct volume was evaluated. The second one is an in vitro model of the same experiment, where primary cortical neurons are deprived temporarily from oxygen and glucose and cell death is evaluated. During ischemia, glucose availability is limited and lack of oxygen impairs oxidative phosphorylation, which produces a significant drop in ATP generation and increases the reduced state of the components of the electron transport chain (Moncada and Erusalimsky, 2002). In these conditions, cytosolic calcium
increases as a consequence of impaired ionic balance and leads to excessive release of glutamate (Halestrap, 2006; Sims and Muyderman, 2010). On reperfusion, oxygen reacts with the reduced components of the electron transport chain and produces a burst in reactive oxygen species (Chinopoulos and Adam-Vizi, 2006; Fiskum et al, 2004). Oxygen also restores mitochondrial membrane potential, which in turn promotes calcium entry to the mitochondrial matrix via the mitochondrial calcium uniporter. All these events lead to PTP opening and cell death (Crompton, 1999).

### 3.4.1. SCaMC-3<sup>−/−</sup> mice show reduced infarct size after middle cerebral arterial occlusion.

The middle cerebral artery is one of the main vessels that supplies blood to the brain and blockade of blood flow through it has been used in the last decades as a model of both reversible (Longa et al, 1989; Nagasawa and Kogure, 1989) and irreversible (Chen et al, 1986; Tamura et al, 1981) focal cerebral ischemia. Importantly, occlusion of the middle cerebral arterial (MCAo) mimics the most common situation in human stroke, which has made this model an indispensable tool for clinical studies (Durukan and Tatlisumak, 2007). The main areas affected by reversible MCAo are the striatum and the cortex, with the extent of the injury being highly correlated, in the case of reversible ischemia, with the time of occlusion (Nagasawa and Kogure, 1989; Popp et al, 2009).

Wild type and SCaMC-3<sup>−/−</sup> 3-month old male mice (n=4-7) were subjected to MCAo using two different time lengths of occlusion, 60 and 90 minutes. The surgical procedure was performed as indicated in Materials and methods. Mice were sacrificed 48 hours after MCAo and the brains were removed, sectioned and stained with H&E to analyse the infarct volume (Figure 3.18A). In addition, both at 24 and 48 hours after occlusion, five kinetic neurological tests were performed to functionally confirm the extent of cerebral damage, namely body symmetry, gait, circling behaviour, front limb symmetry and compulsory circling. A value is assigned for each test depending on the severity of the phenotype and addition of five
values renders a final score ranging 0 (lowest neuronal damage) to 20 (highest neuronal damage) (De Simoni et al, 2003) (see Materials and methods and Table 3.5). As indicated in Table 3.5, animals from both genotypes showed similar weights throughout the experiment, and occlusion of the artery induced the same values of flux drop and flux recovery. Mice lacking SCAcM-3 showed smaller infarct volumes at both 60 minutes of MCAo (8.08 ± 4.04 vs 14.18 ± 3.35 mm³), and at 90 minutes of MCAo (18.76 ± 15.34 vs 41.46 ± 7.89 mm³ in wild type) (Figure 3.18A and Figure 3.18B). The differences in infarct size were significant at the longer time of occlusion (p<0.01 two-way ANOVA followed by Bonferroni multiple comparisons test). Concomitantly, these animals also obtained lower neurological scores 24 and 48 hours (Table 3.5), indicating reduced cerebral damage. We conclude that SCAcM-3⁻/⁻ mice are protected against ischemia/reperfusion in brain, and have lower infarcts and reduced functional cerebral damage, as measured by the neurological tests.

3.4.2. Primary cortical neurons from SCAcM-3⁻/⁻ mice are protected against oxygen and glucose deprivation.

To further study the in vivo results from ischemia/reperfusion we studied oxygen and glucose deprivation.
Table 3.5. Weights, neuronal scores and blood flow values of mice subjected to MCAo. No differences between genotypes were observed concerning weights or blood flow drops and recoveries. *SCaMC-3* animals showed lower neuronal scores after 90 minutes of ischemia, which correlates with a smaller infarct size in these mice. Data shown as mean ± S.E.M. (*p<0.05; unpaired, one-tailed Student t).
(OGD) in primary neuronal cultures obtained from cerebral cortex of wild type and ScaMC-3−/− mice at embryonic day 16 as indicated in Materials and methods. Cultures were used at 10-days in vitro (DIV), exposed to OGD for 1h30 minutes, and analysed for cell death 24 hours later. OGD was attained by changing the cell media (Neurobasal; NB) to glucose-free media (Neurobasal A; NBA), and by introducing the cell plates in a hypoxic chamber at 37°C where oxygen concentration is reduced to 0.6%. Cells from the same cultures were kept in parallel at the same conditions, but with 21% oxygen, in NBA medium supplemented with 25 mM glucose, glucose concentration in NB (Kleman et al, 2008; Russell et al, 2002), and were used as nomoxic controls. Protein expression was examined in the cell cultures to confirm that neither ScaMC-3, ScaMC-1 or ScaMC-2 expressions are affected by OGD, as it has been previously observed that the expression of several mitochondrial carriers in humans is affected by ischemia (Lukk et al, 2010), and no differences were observed between the protein levels during normoxia and OGD (Figure 3.19A). In parallel to the in vivo situation, ScaMC-3-deficient cultures appear to be more resistant to OGD, with lower rates of cell death measured by calcein/PI (Figure 3.19B). In wild type neurons, the OGD/nomoxic death ratio was 1.76 ± 0.18 compared to 1.58 ± 0.14 in ScaMC-3-deficient neurons (p=0.015, paired, one-tailed Student t) and OGD-induced death was also lower in ScaMC-3-deficient cultures (70 ± 8% in wild type cells compared to 55 ± 8% in ScaMC-3-deficient cells, p=0.04 paired, one-tailed Student t). Although neurons from ScaMC-3−/− cultures appear to be more resistant to cell death induced by OGD, the effect seems to be somewhat less pronounced that in vivo. A reason for this may be related to the substantial basal cell death observed in our cultures at 10 DIV, ranging 35-45%.

**Figure 3.19. Reduced cell death in primary cortical neurons from ScaMC-3−/− mice after OGD.** A. Western blot using 10 μg of total cell lysates from wild type (wt) and ScaMC-3−/− (ko) primary cortical neurons 24 hours after OGD or normoxia. Expression of β-ATPase was used as loading control. ScaMC-1, ScaMC-2 and ScaMC-3 showed similar levels in both conditions, indicating absence of up-regulation of these proteins by OGD. B. Cell death 24h after OGD in wild type and ScaMC-3-deficient primary cortical neurons measured as the percentage of dead cells (i.e. stained with 1 μM PI) respect total cells (i.e. stained with PI and stained with 2 μM calcein, which stains living cells). (n=8; *p<0.05; paired, one-tailed Student t)
DISCUSSION
Although the activity and regulation of the mitochondrial ATP-Mg/Pi carrier were extensively studied during the 80s and 90s (Aprille, 1981; Aprille et al, 1982; Dransfield and Aprille, 1993; Dransfield and Aprille, 1994; Hagen et al, 1993; Hagen et al, 2003; Joyal and Aprille, 1992; Joyal et al, 1995; Nosek et al, 1990), its molecular identity has remained elusive until recent years. The identification of the coding genes for the carrier has revealed an unexpected complexity, with five different paralogs described to date and several splicing forms (Amigo et al, 2012; Del Arco, 2005; Del Arco and Satrústegui, 2004; Fiermonte et al, 2004; Haitina et al, 2006; Traba et al, 2009b) (reviewed in Satrústegui et al, 2007b and Traba et al, 2011). We have now evaluated the role of SCaMC-3 in liver and brain using SCaMC-3-deficient mice. In liver, the tissue were the mitochondrial ATP-Mg/Pi carrier has been more thoroughly studied, we have analysed the contribution of SCaMC-3 to paradigms where the carrier has been described to be important, such as the mitochondrial uptake of adenine nucleotides and the regulation of gluconeogenesis, ureogenesis and oxidative phosphorylation, whereas in brain we have evaluated the different susceptibility of SCaMC-3−/− mice to KA toxicity and ischemia/reperfusion injury.

1. The kinetics of SCaMC-3

The available data regarding the transport kinetics of SCaMC-1 and SCaMC-3, obtained by measuring homo-exchanges using reconstituted proteoliposomes, indicate that both proteins have similar Km values for ATP-Mg (~0.2 mM) and Pi (~1.5 mM), but that SCaMC-1 has a lower Km for ADP (~0.3 mM vs ~0.5 mM for SCaMC-3) (Fiermonte et al, 2004). The most significant differences arise from calculated Vmax, with SCaMC-1 showing ~5–7.5-fold higher rates compared to SCaMC-3 (~350-530 μmol/min/g protein vs ~70 μmol/min/g protein) (Fiermonte et al, 2004). As for the calcium dependency, permeabilised COS-7 cells, which express high levels of SCaMC-1, show half maximal activation of ATP-Mg uptake at 12.7 ± 5.3 μM calcium (Traba et al, 2012), whereas isolated mitochondria from mouse liver, where SCaMC-3 is the only functional paralog, show an S0.5 for calcium activation of 3.3 ± 0.9 μM calcium (Amigo et al, 2013). Therefore, two different kinetic and regulatory patterns emerge: on one hand, SCaMC-1 can potentially achieve faster rates of transport, but has higher calcium requirements, while on the other, SCaMC-3 is activated at lower calcium concentrations, although with a lower transport rate.

2. Role of SCaMC-3 in the actions of glucagon in liver mitochondria.

Glucagon and calcium-mobilising agents increase the adenine nucleotide content from liver mitochondria (Aprille et al, 1982; Aprille et al, 1987; Haynes et al, 1986; Hensgens et al, 1980). The exact mechanism by which adenine nucleotides are taken into the mitochondria is not well understood. According to Halestrap and collaborators, calcium entry in the mitochondria could inhibit the mitochondrial pyrophosphatase and accumulated pyrophosphate be exchanged by cytosolic adenine nucleotides through the adenine nucleotide translocator (Davidson and Halestrap, 1988). An alternative mechanism involves the ATP-Mg/Pi carrier, which can increase the mitochondrial adenine nucleotide pool after its activation by cytosolic calcium (Aprille et al, 1982; Nosek et al, 1990). Our results strongly argue in favour of the
second possibility, as mice lacking SCaMC-3 fail to increase the levels of adenine nucleotides in liver mitochondria after glucagon treatment (Figure 3.10B).

However, the extent to which the increase in mitochondrial adenine nucleotides observed after glucagon treatment regulates oxidative phosphorylation is not clear. Asimakis and collaborators using isolated mitochondria showed a biphasic linear relationship between mitochondrial adenine nucleotide content and state 3 respiration (Asimakis and Aprille, 1980a). At low matrix adenine nucleotide concentrations (below 4 nmol/mg of protein), small changes induce big respiratory alterations, whereas this effect is not so steep at higher (4-10 nmol/mg protein) concentrations. Moreover, previous observations indicate that mitochondria can loose over 70% of their adenine-nucleotide content without decreasing their respiratory rates (Meisner and Klingenberg, 1968). Our results using isolated mitochondria indicate a similar behaviour (Figure 3.5). Although we have not measured directly the adenine nucleotide content in mitochondria depleted and refilled with adenine nucleotides, we have observed a linear correlation between respiratory control ratios and low times (0-2 minutes) of incubation of adenine nucleotide-depleted mitochondria with ATP-Mg. After 2 minutes of incubation, respiratory control ratios reach a plateau and is not increased by longer incubation times (Figure 3.5A). Moreover, intact mitochondria incubated with ATP also fail to increase their respiratory rates (Figure 3.5B), suggesting that respiratory control of mitochondrial respiration by intramitochondrial adenine nucleotides is only relevant at low matrix adenine nucleotide levels, when these become rate-limiting, a situation similar to the one observed in newborn liver mitochondria and after compromised energy availability (Watanabe et al, 1983).

Nevertheless, our results using primary hepatocytes and in vivo indicate that SCaMC-3 can effectively mediate an increase in mitochondrial coupled respiration and maximum mitochondrial respiratory capacity after stimulation with glucagon and, in the case of isolated hepatocytes, with phenylephrine (Figures 3.9 and 3.10), agonists that increase the adenine nucleotide levels in the liver mitochondrial matrix (Figure 3.10B and Aprille et al, 1982; Hensgens et al, 1980; Siess et al, 1977). Quantification of mitochondrial adenine nucleotide levels in liver mitochondria in vivo indicates that the total pool of adenine nucleotides increases 2-fold, from 10 nmol/mg protein to around 20 nmol/mg protein after glucagon treatment (Figure 3.10B). These values are well above the range where Asimakis and Aprille fail to see a linear relation with mitochondrial respiration (Asimakis and Aprille, 1980a), suggesting that the uptake of adenine nucleotides brought about by glucagon (and dependent on SCaMC-3) is insufficient to cause the full increase in mitochondrial respiration observed. In other words, SCaMC-3 appears to be permissive for other actions of glucagon in mitochondria. Additional events that are not reproduced in isolated liver mitochondria must take place in vivo, and it is feasible that the stimulation of respiration observed is due to a yet unidentified effect of mitochondrial adenine nucleotides on mitochondrial respiration, rather than by a direct mass action effect on the ANT or the F1-ATP synthase.
The stimulation of respiration by glucagon has been the subject of a great amount of work during the decades of the 70s and 80s, although the site of action of the hormone remains unknown. The enhancement of oxygen consumption is observed when the electron transport chain is fuelled with substrates of complex I or complex II, but not when TMPD plus ascorbate are used instead (Yamazaki, 1975). It has been concluded that glucagon-induced activation of respiration involves increases in electron flow into the ubiquinone pool and within complex III (Halestrap, 1982; Halestrap, 1989). The mechanism proposed for such stimulation is a moderate increase in matrix volume caused by the uptake of calcium into the mitochondria following glucagon-induced cytosolic calcium signals. By inhibiting mitochondrial pyrophosphatase activity matrix calcium would cause an increase in pyrophosphate and an influx of K\(^+\) into the mitochondria through a K\(^+\) uniporter whose nature is still debated (Garlid and Halestrap, 2012).

Both glucagon and calcium-mobilising promote an increase in mitochondrial levels of calcium that parallels that of mitochondrial adenine nucleotides (Assimacopoulos-Jeannet et al, 1986). These hormones also promote an increase in the mitochondrial CRC (Prpić et al, 1978). Adenine nucleotides are required within the matrix to accumulate large amounts of calcium through the formation of calcium-phosphate precipitates (Carafoli et al, 1965 and see Introduction). Our results show that the effect of glucagon in CRC do not take place in the absence of SCaMC-3 (Figure 3.11D), further supporting the idea that the ATP-Mg/Pi carrier plays a crucial role calcium retention by mitochondria (Traba et al, 2012 and see below). Ineffective calcium retention in mitochondria could also be to some extent responsible for the absence of stimulation of respiration by glucagon in SCaMC-3-deficient, as calcium is regarded as the main regulator of mitochondrial respiration (Glancy and Balaban, 2012). Although we have failed to detect an increase in total calcium (measured by atomic absorption) in liver mitochondria isolated from glucagon treated mice (not shown), other authors have described such an increase (Assimacopoulos-Jeannet et al, 1986), and matrix calcium could underlie the increase in state 3 respiration (through calcium effects on ATP-synthase) or in respiration with complex I substrates (through calcium activation of matrix dehydrogenases) in mitochondria from glucagon-treated animals. Alternatively, failure to increase mitochondrial calcium concentration in vivo during glucagon treatment may result in a lack of change in matrix volume, which could itself be responsible for the absence of stimulation of respiration in mitochondria from SCaMC-3-deficient animals. Further studies are required to elucidate the mechanism by which glucagon increases respiration in liver mitochondria and the precise role of SCaMC-3.

It should be noted that calcium retained in the mitochondria have a complex influence on mitochondrial function. On one hand, calcium is necessary to activate dehydrogenases and ATP synthase, and lowering matrix free calcium levels with mitochondrially targeted DNA-coded calcium buffers results in the lost of calcium activation of mitochondrial function in insulin-secreting beta cells (Wiederkehr et al, 2011).
Probably, a certain threshold of matrix free calcium concentration must also be met to trigger the mild volume increases which are thought to activate liver mitochondrial respiration in response to glucagon. Although matrix free calcium levels in SCaMC-3-deficient hepatocytes have not been studied, it is possible that conditions favouring ATP-Mg uptake in mitochondria will result in lower total calcium accumulation and higher matrix free calcium levels, as shown for SCaMC-1 deficient cells (Traba et al, 2012). Higher matrix free Ca levels in response to glucagon may perhaps compensate to some extent for the lack of increase in matrix adenine nucleotides in regulating respiration. But most importantly, a further increase in matrix free calcium in the face of a lower CRC would clearly switch a mild increase in volume connected with stimulation of respiration to a larger swelling related to PTP opening and inhibition of respiration. Halestrap's work has shown in intact hepatocytes that the increase in mitochondrial volume caused by phenylephrine is larger than that caused by glucagon, possibly in relation the sizes of the calcium signals elicited by both agonists (McCormack et al, 1990). Interestingly, SCaMC-3 deficient hepatocytes increase the proton leak and decrease maximal respiration in response to phenylephrine, suggestive of a mitochondrial imairment probably related to PTP opening. Accordingly, we have found evidence of liver failure in SCaMC-3-/- mice, reflected in the release of liver markers to the bloodstream in these mice (Table 3.2). Our results suggest that PTP opening events resulting in mitochondria dysfunction may be responsible for this liver failure.

3. Role of SCaMC-3 in other liver functions

It has been suggested that the increase in matrix adenine nucleotide levels is important for the activity of ATP-dependent enzymes, such as pyruvate carboxylase, which is critical for gluconeogenesis (Aprille et al, 1981), and carbamoyl phosphate synthetase I (CPSI), which requires two ATPs for the synthesis of carbamoyl phosphate, and is critical for citrulline and urea synthesis (Goldstein and Aprille, 1982). We have observed a significant decrease in plasma urea levels but no changes in glucose levels in SCaMC-3-/- mice. This may suggest that failure to increase matrix ATP via SCaMC-3 may impair urea synthesis. However, the ATP requirements of pyruvate carboxylase and CPSI are similar. The Km for ATP in pyruvate carboxylase is about 0.5 mM (Scrutton and Utter, 1965) and that of CPSI for each of the two ATP molecules it binds is 10-20 μM (Rubio et al, 1979) and 0.5-0.8 mM (Elliot and Tipton, 1974) respectively. On the other hand, CPSI concentration is close to 20% of the liver mitochondrial protein (Jackson et al, 1986), suggesting that CPS1 may impose a heavier load on ATP utilization in mitochondria, which may become limited by ATP-Mg supply through SCaMC-3.

4. Role of SCaMC-3 in mitochondrial calcium-overload paradigms

Recent work from our laboratory has shown that the ATP-Mg/Pi carrier is required to effectively buffer mitochondrial matrix calcium and that, in its absence, cells are more susceptible to stimuli that induce calcium-overload (Traba et al, 2012). Moreover, we have observed that SCaMC-3-deficient mitochondria
from liver and brain accumulate less calcium in the presence of adenine nucleotides (and hence are more prone to undergo PT), and that primary cortical neurons from SCaMC-3<sup>−/−</sup> mice show increased sensitivity to glutamate-induced cell death and reduced mitochondrial calcium buffering (unpublished results). To test these observations in more physiological conditions, we studied the effect of the absence of SCaMC-3 in two well established paradigms of <i>in vivo</i> calcium overload: KA administration and ischemia/reperfusion injury (Li et al, 2009; Nakagawa et al, 2005; Schinzel et al, 2005).

Administration of KA induced early seizure appearance, more severe convulsions and higher astrogliosis response in SCaMC-3<sup>−/−</sup> mice, but we failed to observe cell death in the hippocampus following the treatment (Figures 3.16 and 3.17). Although seizure appearance and brain cell death are commonly associated (Krajewska et al, 2011; Tripathi et al, 2009; Wu et al, 2005), a causal link between both events has not been established. It is now clear that the relative contribution of each of these events to the KA response is largely dependent on the genetic background of the mice used in the experiments (McKhann et al, 2003; McLin and Steward, 2006; McLin et al, 2006). The strain used in our experiments, C57BL6/Sv129, has been shown to be prone to seizure appearance, but relatively resistant to histopathological alterations and cell death, which would explain our observations (McKhann et al, 2003).

As for seizures and brain inflammation, these events appear to be more interrelated (Bartfai et al, 2007; Vezzani and Granata, 2005). Seizures seem to be originated by a pathological increase in excitatory neurotransmission (Zucchiini et al, 2008). In the case of the recently described HMGB1-TLR4 pathway, whose activation by KA triggers inflammation and cytokine release by brain cells, its proconvulsant effect has been explained by phosphorylation of NR2B-containing NMDA receptors, increasing calcium uptake in neurons (Maroso et al, 2010). It seems probable that this increase in cytosolic calcium induces a subsequent increase in mitochondrial calcium. In fact, KA-induced seizures have been shown to promote mitochondrial oxidative stress (Liang and Patel, 2006). Therefore, defective mitochondrial calcium buffering in SCaMC-3<sup>−/−</sup> cells could account for the augmented seizure phenotype observed in mutant mice, consistent with previous observations on increased oxidative stress in cells lacking the ATP-Mg/Pi carrier (Traba et al, 2012).

Regarding ischemia/reperfusion, mice lacking SCaMC-3 show reduced cell death in both <i>in vivo</i> and <i>in vitro</i> models (Figures 3.18 and 3.19), which may seem paradoxical, given the fact that low levels of expression of the ATP-Mg/Pi carrier affect negatively the mitochondrial CRC (see above). However, lack of oxygen during ischemia or OGD is an important parameter for ATP-Mg/Pi activity. Low tension of oxygen reduces electron transport chain activity, decreasing mitochondrial membrane potential and proton motive force, while lack of glucose induces a low energetic state in the cytoplasm and a cytosolic calcium increase (Sims and Muyderman, 2010). In these conditions, efflux of adenine nucleotide through the
mitochondria has been described through two non mutually exclusive mechanisms. First, during ischemia, lack of energy in the mitochondria promotes degradation of all three adenine nucleotide forms, ATP, ADP and AMP, to adenosine, which is leaked into the cytoplasm (Watanabe et al, 1983). Second, cytosolic calcium, mitochondrial proton motive force and gradients of Pi and adenine nucleotides favour activation of the ATP-Mg/Pi towards a net efflux of adenine nucleotide from the matrix (Aprille, 1993; Hagen et al, 1993).

It is reasonable to believe that absence of this adenine nucleotide efflux through the ATP-Mg/Pi carrier in SCaMC-3+/− mice could underlie the protection observed after ischemia/reperfusion injury. Maintenance of a mitochondrial adenine nucleotide pool could be beneficial for the cell for several reasons. First, keeping the adenine nucleotides within the mitochondria could prevent total hydrolysis in the cytoplasm by membrane ion pumps, whose activity is extremely high in hypoxic conditions, and facilitate recovery on reperfusion (Dransfield and Aprille, 1994). Second, in ischemia/reperfusion models mitochondrial calcium overload is believed to take place during reperfusion, when recovery of mitochondrial membrane potential allows calcium uptake through the MCU (Baines, 2009a). Avoiding the efflux of mitochondrial adenine nucleotides during ischemia would increase CRC on reperfusion, as both factors are positively correlated, and prevent/delay PT and subsequent cell death (Chinopoulos and Adam-Vizi, 2010; Traba et al, 2012). Third, protection could be afforded by phosphorylation of mitochondrial targets that reduce oxidative phosphorylation and reactive oxygen species production, due to relatively high levels of ATP in the matrix of SCaMC-3+/− cells after ischemia. Possible candidate targets include the pyruvate dehydrogenase complex (Hitsugiyi et al, 2011) and cytochrome oxidase (Acin-Perez et al, 2011). In this line of evidence, activity of mitochondrial kinases such as PKA (Pediatitakis et al, 2010) and PDHK1 (Hitsugiyi et al, 2011) has been reported to be protective in similar conditions.
CONCLUSIONS
1. SCaMC-3 is the main form of the mitochondrial carrier of ATP-Mg/Pi in liver.

2. Plasma urea levels are decreased in SCaMC-3−/− mice, suggesting a possible defect in urea synthesis associated with the absence of the transporter.

3. Lack of SCaMC-3 results in an increase in liver markers in the plasma, indicating liver injury.

4. SCaMC-3 deficiency does not affect basal respiration of liver mitochondria.

5. After depletion of adenine nucleotides, liver mitochondria take up ATP-Mg or ADP through SCaMC-3 in the presence, but not in the absence of calcium. Until a certain threshold of adenine nucleotides, this uptake correlates with an increase in coupled respiration, probably through mass action effects on the ADP/ATP translocase or the ATP synthase.

6. SCaMC-3 is responsible for the increase in adenine nucleotide content and calcium retention capacity observed in liver mitochondria after glucagon treatment. These events might be causally correlated, as adenine nucleotide import into the matrix has been shown to be necessary for effective calcium retention.

7. Glucagon stimulates coupled respiration, both in vivo and in isolated hepatocytes, through a mechanism that requires SCaMC-3.

8. SCaMC-3−/− mice are more susceptible to seizures and inflammatory response after excitotoxic damage caused by kainic acid. However, absence of SCaMC-3 is protective against middle arterial cerebral occlusion and oxygen and glucose deprivation. Based on the full reversibility of the ATP-Mg/Pi exchange reaction, it is suggested that SCaMC-3 might be promoting the uptake of adenine nucleotides during excitotoxicity, and hence preventing permeability transition pore opening, but favouring matrix adenine nucleotide loss during ischemia, contributing to cell death on reperfusion.
RESUMEN EN ESPAÑOL
1. INTRODUCCIÓN

Los transportadores mitocondriales son proteínas de la membrana mitocondrial interna que permiten el intercambio de solutos entre el citoplasma de la célula y la matriz mitocondrial. Su estructura se caracteriza por tres repeticiones de unos 100 aminoácidos, cada una con dos dominios transmembrana unidos por una secuencia hidrofílica, y la presencia de una secuencia característica altamente conservada. Actualmente se conocen unos 50 transportadores en humanos que se encuentran agrupados en la familia SLC25 (Palmieri et al, 2006).

Dentro de la familia de transportadores mitocondriales existe un subgrupo caracterizado por la presencia de dominios de unión a calcio de tipo manos EF en su extremo N-terminal, orientados hacia el espacio intermembrana. Este subgrupo se divide a su vez en dos tipos: los transportadores de aspartato-glutamato y los transportadores de ATP-Mg/Pi (revisado en Satrústegui et al, 2007b).

Los transportadores de ATP-Mg/Pi, también denominados SCaMCs (acrónimo de short calcium- binding mitochondrial carriers), se describieron durante los años 80, aunque los genes y proteínas responsables por el transporte no fueron identificados hasta 2004 (Del Arco y Satrústegui, 2004; Fiermonte et al, 2004). Catalizan el intercambio no electrogénico entre ATP-Mg\(^2\) o HADP\(^-2\) y HPO\(_4\)\(^-2\) y requieren la presencia de calcio citosólico del orden micromolar para su actividad. Mediante este transporte pueden alterar la cantidad neta de nucleótidos de adenina y potencialmente regular reacciones de la matriz mitocondrial que requieran ATP o ADP. La orientación del transporte está condicionada por el gradiente de concentración de los sustratos e indirectamente por el potencial de membrana mitocondrial. En mamíferos se han descrito cinco parálogos diferentes, denominados SCaMC-1, SCaMC-2, SCaMC-3, SCaMC-1like y SCaMC-3like.

En hígado y cerebro la forma mayoritaria es SCaMC-3. Mediante el empleo de ratones knock-out para el gen que codifica SCaMC-3, hemos caracterizado la algunas de las funciones de esta proteína en ambos tejidos.
2. OBJETIVOS

El objetivo principal del presente trabajo es la caracterización del papel de SCaMC-3, el principal parálogo del transportador de ATP-Mg/Pi en hígado y cerebro, tanto en condiciones fisiológicas como patológicas. En cuanto a las primeras, el transportador se ha descrito como una posible diana de la señalización de glucagon en la mitocondria hepática, importante en la regulación de la gluconeogenesis y la ureogenesis. El transportador también ha sido implicado en el llenado de nucleótidos de adenina que tiene lugar en la mitocondria de hígado durante las primeras horas post-natales. Con respecto al papel de SCaMC-3 en la muerte celular, publicaciones recientes han implicado al transportador de ATP-Mg/Pi en la regulación de la retención de calcio en la mitocondria, un evento clave en la señalización celular y cuyos defectos han sido relacionados con diversas condiciones patológicas, incluidas la isquemia y reperfusión y la excitotoxicidad. Por lo tanto, nos hemos planteado tres objetivos principales para el presente trabajo:

1. Descripción de las características generales de los ratones SCaMC-3/− y de las mitocondrias deficientes en SCaMC-3.

   a) Análisis de los diferentes parálogos del transportador de ATP-Mg/Pi en diferentes tejidos y etapas de desarrollo.
   b) Efecto de la ausencia de SCaMC-3 en los niveles de glucosa y urea en respuesta al ayuno.
   c) Evaluación de los defectos putativos en la respiración mitocondriales debidos a la ausencia de SCaMC-3.

2. Papel de SCaMC-3 en la respiración de las mitocondrias hepáticas en respuesta a glucagon.

   a) Papel de SCaMC-3 en la fosforilación oxidativa en mitocondrias vaciadas de nucleótidos de adenina.
   b) Efecto del glucagon in vivo en los niveles de nucleótidos de adenina, respiración y capacidad de retención de calcio en mitocondrias de hígado y en hepatocitos deficientes en SCaMC-3.

3. Análisis del efecto de la ausencia de SCaMC-3 en modelos de sobrecarga de calcio en cerebro.

   a) Caracterización de la expresión de SCaMC-3 en cerebro.
   b) Efecto de la ausencia de SCaMC-3 en el daño provocado por ácido kainico.
   c) Efecto de la ausencia de SCaMC-3 en modelos in vivo e in vitro de isquemia/reperfusión.
3. RESULTADOS

Los ratones deficientes en SCaMC-3 nacen en proporciones mendelianas y no presentan alteraciones fenotípicas. La función respiratoria de sus mitocondrias en hígado y cerebro es normal, con tasas similares a las de los ratones *wild type* con sustratos de complejo I y II y niveles equivalentes de masa mitocondrial, medidos como la actividad citrato sintasa.

Cuando las mitocondrias se vacían de nucleótidos de adenina mediante incubación con pirofosfato la respiración mitocondrial deja de poder estimularse con ADP y se requiere el transporte neto de nucleótidos para recuperar dicho control respiratorio. Nuestros resultados indican que, en estas circunstancias, la adición de ADP o la incubación con ATP son capaces de recuperar el acoplamiento entre respiración y síntesis de ATP de forma dependiente de la presencia de SCaMC-3 y de calcio. Asimismo, hemos visto que la presencia de SCaMC-3 es necesaria para la correcta transducción de la señal de glucagon en las mitocondrias de hígado. Las mitocondrias de ratones deficientes en la proteína muestran ausencia de captación de nucleótidos de adenina y una disminución en la estimulación tanto en la respiración como en la capacidad de retención de calcio, en respuesta al tratamiento de los ratones con glucagon.

En cerebro hemos visto que la falta de SCaMC-3 hace más susceptibles a los ratones frente a la excitotoxicidad producida por ácido kainico. Concretamente, los ratones deficientes en SCaMC-3 muestran un fenotipo convulsivo más severo y una mayor respuesta inflamatoria. Por otro lado, y de forma inesperada, la ausencia de SCaMC-3 disminuye el tamaño de infarto como resultado de la oclusión de la arteria cerebral media, y la muerte celular en respuesta a la deprivación de glucosa y oxígeno en cultivos primarios de neuronas corticales.
4. DISCUSIÓN

El glucagon es una hormona producida por el páncreas durante el ayuno y que en el hígado estimula procesos catabólicos como la glicólisis y la degradación de glucógeno a glucosa, en detrimento de rutas anabólicas como la síntesis de glucógeno y la gluconeogenesis. En las mitocondrias hepáticas produce, entre otros efectos, un aumento de los niveles totales de nucleótidos de adenosina, una estimulación de la respiración y un incremento de la capacidad de retención de calcio. Nuestros resultados indican que SCaMC-3 interviene en estos tres procesos. En cuanto al aumento de los niveles de nucleótidos de adenosina, un proceso que requiere calcio, se barajaban dos posibles mecanismos: Halestrap y colaboradores propusieron que el aumento de calcio citósolico producido por el glucagon generaría a su vez un aumento en el calcio mitocondrial que inhibiría a la pirofosfatasa mitocondrial, produciéndose un aumento en los niveles de pirofosfato en la matriz. Este pirofosfato podría ser intercambiado por nucleótidos de adenosina de la matriz mitocondrial a través de la ATP/ADP translocasa. Por otro lado, Aprille y colaboradores propusieron que el calcio citósolico podría actuar directamente activando al transportador de ATP-Mg/Pi, que sería el responsable del incremento de nucleótidos de adenosina en la matriz mitocondrial. Nuestros resultados respaldan esta última hipótesis, ya que las mitocondrias hepáticas de los ratones deficientes en SCaMC-3 no incrementan sus niveles de nucleótidos de adenosina en respuesta a glucagon. A su vez, estas mitocondrias tampoco son capaces de estimular la respiración después de tratamiento con la hormona, aunque no hemos sido capaces de establecer un vínculo causal entre ambos fenómenos. En cuanto al incremento de la captura de calcio, el transporte de ATP-Mg y ADP a través de SCaMC-3 es responsable de un mejor tamponamiento del calcio mitocondrial, posiblemente por permitir la formación de precipitados de calcio-fosfato en la matriz mitocondrial.

En cerebro, hemos observado que la ausencia de SCaMC-3 protege frente a depredación de oxígeno y glucosa, pero aumenta la susceptibilidad al daño por ácido kainico, en ambos casos modelos de muerte que cursan con incrementos tóxicos de calcio citósolico. En el primer caso, hipotetizamos que la falta de oxígeno y la consiguiente disminución del potencial de membrana mitocondrial, unida a la caída en los niveles de ATP citósolico, podrían favorecer la salida de nucleótidos de la mitocondria hacia el citosol a través de SCaMC-3, haciendo más susceptibles a las neuronas a la sobrecarga de calcio. Finalmente, en el caso del kainato, SCaMC-3 funcionaría introduciendo nucleótidos en la matriz, favoreciendo el tamponamiento de calcio por la mitocondria y protegiendo a la célula.
4. CONCLUSIONES

1. SCaMC-3 es el parálogo principal del transportador de ATP-Mg/Pi en hígado.

2. Los niveles basales de urea en plasma se encuentran reducidos en ratones deficientes en SCaMC-3, indicando un posible defecto en la síntesis de urea asociado a la falta del transportador.

3. La falta de SCaMC-3 produce un aumento de marcadores de hígado en plasma, indicadores de daño hepático.

4. Los niveles de respiracion basal de las mitocondrias de hígado no se ven afectados por la deficiencia de SCaMC-3.

5. Las mitocondrias de hígado vaciadas de nucleótidos de adenina captan ATP-Mg o ADP a través de SCaMC-3 en presencia, pero no en ausencia, de calcio. Hasta un valor umbral de nucleótidos, este transporte correlaciona con un aumento en la respiración acoplada, probablemente por efecto de acción de masas de los nucleótidos sobre la ADP/ATP translocasa o la ATP sintasa.

6. SCaMC-3 es responsable por el aumento del contenido de nucleótidos de adenina y la capacidad de retención de calcio que tiene lugar en mitocondrias hepáticas con el tratamiento con glucagon. Ambos eventos pueden estar relacionados causalmente, ya que la captación de nucleótidos de adenina a la matriz es necesaria para la retención de calcio en la mitocondria.

7. El glucagon estimula la respiración acoplada tanto in vivo como en hepatocitos aislados, mediante un mecanismo que requiere SCaMC-3.

8. Los ratones deficientes en SCaMC-3 son más susceptibles a la aparición de convulsiones y tienen una respuesta inflamatoria exacerbada en respuesta al tratamiento con kainato. Sin embargo, están protegidos frente al daño isquémico y la deprivación de glucosa y oxígeno. Es probable que este comportamiento diferencial en ambos modelos sea consecuencia de la reversibilidad de la reacción catalizada por SCaMC-3.
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APPENDIX