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Mini-Review

Role of Aralar, the Mitochondrial Transporter of Aspartate-Glutamate, in Brain N-Acetylaspartate Formation and Ca^{2+} Signaling in Neuronal Mitochondria

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Aralar, the Ca^{2+}-dependent mitochondrial aspartate-glutamate carrier expressed in brain and skeletal muscle, is a member of the malate–aspartate NADH shuttle. Disrupting the gene for aralar, *SLC25a12*, in mice has enabled the discovery of two new roles of this carrier. On the one hand, it is required for synthesis of brain aspartate and N-acetylaspartate, a neuron-born metabolite that supplies acetate for myelin lipid synthesis; and on the other, it is essential for the transmission of small Ca^{2+} signals to mitochondria via an increase in mitochondrial NADH.

AQ2 **Key words: ●●●**

Calcium-binding mitochondrial carriers (CaMCs) comprise a family of mitochondrial transporters with N-terminal extensions that harboring a number of EF-hands, the archetypical calcium-binding motifs (Fig. 1). F1 There are two types of CaMCs, aspartate-glutamate carriers (AGCs) and ATP-Mg/Pi carriers. The activity of these two carriers has been known for more than 20 years, thanks to the pioneering research in AGCs by Kay LaNoue and in ATP-Mg/Pi carriers by June Aprille (LaNoue and Schoolwerth, 1979; Aprille, 1993).

In recent years the genes and proteins responsible for these activities have been identified. Humans have two AGCs, aralar (also called aralar1; del Arco and Satrústegui, 1998) and citrin (Kobayashi et al., 1999; del Arco et al., 2000), encoded by genes on chromosomes 2q31 (Sanz et al., 2000) and 7q21 (Sinasac et al., 1999), respectively, and three ATP-Mg/Pi carriers that are short CaMCs, SCaMC 1–SCaMC 3 (del Arco and Satrústegui, 2004; Fiermonte et al., 2004; del Arco, 2005). Yeast mitochondria also contain these carriers (Cavero et al., 2003, 2005). However, there is only one AGC, Agc1p, which, unlike mammalian AGCs, does not contain any calcium-binding motifs (Cavero et al., 2003). For a review of both types of CaMCs, see del Arco and Satrústegui (2005) and Satrústegui et al. (2007).

The two mammalian AGCs and yeast Agc1p are members of the malate–aspartate NADH shuttle (MAS); citrin is also a member of the urea cycle (Palmieri et al., 2001; Cavero et al., 2003). The transport reaction catalyzed by AGCs, the exchange of matrix aspartate for external glutamate plus a proton, is unidirectional under normal resting conditions, as it is driven by a proton electrochemical gradient and is a main point of regulation.

Abbreviations used: α-KG, α-ketoglutarate; α-KGDH, α-ketoglutarate dehydrogenase; AGC, aspartate-glutamate carrier; $[Ca^{2+}]_i$, cytosolic free Ca^{2+}; $[Ca^{2+}]_{mit}$, mitochondrial free Ca^{2+}; CaMCs, calcium-binding mitochondrial carriers; CaU, Ca^{2+} uniporter; MAS, malate–aspartate NADH shuttle; NAA, N-acetylaspartate; OGC, α-KG–malate transporter; RR, ruthenium red.

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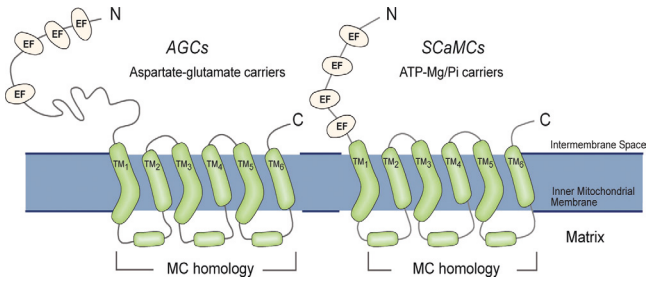

Fig. 1. Secondary structure of calcium-binding mitochondrial carriers (CaMCs). In both CaMCs, the carboxy-terminal half corresponds to the MC homology region, and the N-terminal extension harbors Ca^{2+}-binding EF-hand motifs. The MC homology region, about 300 amino acids long, is represented according to its homology with that of adenine nucleotide translocase in its carboxyatractyloside-bound form (Pebay-Peyroula and Brandolin, 2004). TM1-6 corresponds to the six tilted transmembrane helices characteristic of all MCs, three of which are kinked (1, 3, and 5) because of the presence of conserved prolines. Three shorter helices in the loops between TM 1-2, TM 3-4, and TM 5-6 face the matrix and are parallel with the membrane surface. The MC region forms a basket opening to the intermembrane space, with a funnel-shaped cavity inside that ends on a narrow pit close to the matrix surface (Pebay-Peyroula and Brandolin, 2004). The N-terminal extensions of AGCs are longer than those of SCaMCs, and the overall distribution and sequence of EF-hand motifs is unrelated to that of SCaMCs, which are very similar to calmodulin (reproduced from Satrústegui et al., in press).

The transporter function of AGCs is stimulated by Ca^{2+} acting on the external face of the inner mitochondrial membrane, the same side of the membrane where the Ca^{2+} binding motifs are localized (Palmieri et al., 2001).

This review addresses the new roles of aralar/AGC1, the aspartate–glutamate carrier, in brain and skeletal muscle: first, in the synthesis of brain aspartate and N-acetylaspartate and, second, as a newly discovered mechanism for transmitting small Ca^{2+} signals to neuronal mitochondria. Both these roles were discovered through the development of aralar-deficient mice (Jalil et al., 2005; Pardo et al., 2006).

BRAIN ASPARTATE AND N-ACETYLASPARTATE

Aralar$^{(-/-)}$ mouse embryos are produced in normal numbers, as expected from the overlap of aralar and citrin expression in fetal tissues (Begum et al., 2002; del Arco et al., 2002). However, soon after birth these mice develop severe growth defects and die about 20 days postnatal (Jalil et al., 2005).

As predicted from the known role of AGCs in the MAS, the deficiency in aralar leads to a dose-dependent decrease in shuttle activity in brain and skeletal muscle mitochondria (Jalil et al., 2005). In fact, the activity of mitochondria in *aralar*$^{(-/-)}$ mice is extremely low, indicating that no other glutamate carrier can substitute for aralar in these tissues. However, respiration on glutamate plus malate (but not in pyruvate plus malate or succinate) is drastically reduced but still detectable in skeletal muscle mitochondria. The decreased respiration caused by aralar deficiency confirms the long-standing view that respiration on these two substrates takes place mainly through the AGC as the glutamate transporter (Balazs, 1965). However, as respiration is still detectable in the absence of aralar, other glutamate carriers such as the glutamate–hydroxyl carrier (Fiermonte et al., 2002) may substitute for the AGC in transporting glutamate for mitochondrial respiration.

Aralar$^{(-/-)}$ mice develop motor coordination defects from day 12 onward (Jalil et al., 2005). There are no gross changes in neuronal number or distribution in the brains of *aralar*$^{(-/-)}$ mice. However, these animals show a marked decrease in myelin and myelinated fiber tracts across the central nervous system (CNS) but not in the peripheral nervous system. This is somewhat surprising because aralar is mainly expressed in neurons in the adult CNS (Ramos et al., 2003). Indeed, in situ hybridization studies in mouse brain showed that *aralar* expression was restricted to neuron-rich areas, with no detectable expression in white matter, whereas *citrin* mRNA was not detectable anywhere in the adult brain. A similar neuron-restricted distribution of aralar protein was observed in the spinal cord (Ramos et al., 2003). Moreover, aralar levels in mitochondria were much higher in neuronal cultures than in glial cultures, and these levels increased with neuronal maturation in culture (Ramos et al., 2003). The finding of high aralar levels and MAS activity in neurons agrees with earlier findings of high shuttle activity in synaptosomes (Cheeseman and Clark, 1988) and its requirement for lactate utilization (McKenna et al., 1993). However, glial cells actually may express AGCs but at much lower levels than neurons, because even though of citrin and aralar mRNA was not detectable in white matter regions, both aralar and citrin were detected in extracts from glial cell cultures (Ramos et al., 2003). Therefore, although this idea is still controversial (Dienel and Cruz, 2006), it is unlikely that the MAS is a major redox shuttle in brain astrocytes or retinal Müller cells (LaNoue et al., 2001; see McKenna et al., 2006, for a review), but studies of the different glial cell types are required to settle this point.

If neurons are the brain cells with higher aralar expression, why would an aralar deficiency cause oligodendrocytes to fail to synthesize myelin? The response to this paradox is related, at least in part, to the role of aralar in the synthesis of brain aspartate and N-acetylaspartate (NAA).

NAA is thought to play a role in myelin lipid formation by supplying acetyl groups (D'Adamo and Yatsu, 1966; Burri et al., 1991; Mehta and Namboodiri, 1995; Chakraborty et al., 2001). In the adult brain, neurons are the main cell type able to produce NAA (Urenjak et al., 1992, 1993). Chakraborty et al. (2001) showed that myelin lipids of the rat optic system were labeled following intraocular injection of [^{14}C-acetyl]NAA.

Myelin lipid labeling was attributed to uptake of labeled NAA by retinal ganglion cell perikarya followed by axonal transport in the optic nerve and axon-to-myelin transfer to supply acetyl groups for the synthesis of myelin lipids (Chakraborty et al., 2001). Indeed, the labeling pattern of individual myelin lipids differed substantially depending on whether ^{14}C-acetate or [^{14}C-acetyl]NAA served as the intraocular labeled precursor, suggesting that NAA is transferred to myelin and cleaved in oligodendrocytes rather than in neurons (Chakraborty et al., 2001). Within the nervous system the NAA cleavage enzyme aspartoacylase II is restricted primarily to oligodendrocytes (Bhakoo et al., 2001; Kirmani et al., 2003; Namboodiri et al., 2006), and participating in this transfer could be NaC3, a plasma membrane transporter for NAA enriched in astroglia (Sager et al., 1999), formerly known as Na^+-coupled dicarboxylate transporter 3, NaDC3/SDCT2 (Fujita et al., 2005).

Mutations in aspartoacylase II cause Canavan's disease, characterized by a spongy degeneration of the CNS, increased levels of NAA in the brain and body fluids, and an extensive loss of myelin. Aspartoacylase II–deficient mice, generated as a model of Canavan's disease (Matalon et al., 2000), have decreased myelin lipid synthesis and a very drastic reduction (about 80 %) in brain acetate concentrations (Madhavarao et al., 2005), showing that preventing NAA cleavage results in a lack of acetyl groups for myelin lipid synthesis. Although this does not formally prove the existence of transaxonal transfer of NAA from neurons to oligodendrocytes, it is consistent with such a system because neurons are the main producers of NAA in the adult brain. However, other pathways of NAA-dependent myelin lipid synthesis probably exist as oligodendrocyte type 2 astrocyte progenitors are also able to produce NAA (Urenjak et al., 1992, 1993), which may be an important potential source of NAA for myelin lipid synthesis during development. Indeed, these cells also express aspartoacylase II, although at lower levels than do mature oligodendrocytes or type 2 astrocytes (Bhakoo et al., 2001).

Aralar deficiency resulted in a large drop in NAA and aspartate in the brain and in primary neuronal cultures derived from *aralar*$^{(-/-)}$ mice. In addition, these mice showed a drop in myelin lipid concentration and similar reductions in expression of the major proteins in myelin, myelin basic protein and myelin-associated oligodendrocytic basic protein (Jalil et al., 2005). However, the peripheral nervous system (PNS) of *aralar*$^{(-/-)}$ mice did not show decreased expression of galactocerebrosides, the lipid component characteristic of myelin (Jalil et al., 2005). This is interesting because the Schwann cells in the PNS do not express aspartoacylase (Kirmani et al., 2003), the enzyme required to break down NAA; therefore, defects in NAA formation such as those observed in *aralar*$^{(-/-)}$ mice probably do not play a role in myelin lipid synthesis in the PNS.

The fall in the concentration of brain aspartate levels and the CNS pathology in aralar-null mice (Jalil et al., 2005) appear at about the time the blood–brain barrier develops, preventing the entry of aspartate and other amino acids into most regions of the adult brain except for selected circumventricular regions (Price et al., 1984). From the first week of life onward, the brain has to rely on endogenous production of aspartate, which takes place mostly in neurons (Urenjak et al., 1993), the brain cells with the highest aralar expression (Ramos et al., 2003). The fall in brain aspartate levels and the lack of aspartate production in the brain mitochondria of *aralar*$^{(-/-)}$ mice (Jalil et al., 2005) suggest that a major route of aspartate production in the CNS is mitochondrial and that it depends on aralar for aspartate efflux to the cytosol. This route is probably critical in conditions when the cytosolic NADH/NAD ratio is increased because of a lack of MAS activity, as occurs in neurons from *aralar*$^{(-/-)}$ mice (Jalil et al., 2005; Pardo et al., 2006). Indeed, in these conditions the cytosolic oxaloacetate level is expected to decrease, limiting aspartate production in the cytosol via the aspartate aminotransferase reaction, which rapidly equilibrates aspartate and oxaloacetate (Shen, 2005).

NAA is derived from neuronal aspartate, and its synthesis is mainly catalyzed by aspartate-N-acetyltransferase in an enzyme originally described as mitochondrial (Patel and Clark, 1979) but found to be present both in brain mitochondria (Madhavarao et al., 2003; Sambhu et al., 2005) and in microsomes, which have 4- to 5-fold higher activity than mitochondria (Lu et al., 2004). Thus, the fall in NAA in the brain and in cultured neurons from *aralar*$^{(-/-)}$ mice is clearly associated with a defect in aspartate production.

Even though the precise role of NAA in the nervous system is still unsettled (Baslow, 2003), NAA is commonly used in ^{1}H-NMR spectroscopy as a potential diagnostic marker for evaluating neuronal mitochondrial function and neuronal loss or damage (Pan and Takahashi, 2005; Tsai and Coyle, 1995; Clark, 1998). This interpretation is supported by studies describing NAA synthesis in neuronal mitochondria and linking it to oxygen consumption (Patel and Clark, 1979). NAA turnover has been studied by ^{13}C-NMR labeling from different precursors (Kunnecke et al., 1993; Tyson and Sutherland, 1998), and the findings of a recent study of the incorporation of label into both the acetyl and aspartyl groups of NAA suggest that NAA synthesis occurs in a single metabolic compartment, that aspartate is the precursor of NAA, and that both aspartate and NAA synthesis occur in the neuronal compartment (Choi and Gruetter, 2004). The findings of the study of *aralar*$^{(-/-)}$ mice that attributed the drop in NAA to a defect in neuronal aspartate production (Jalil et al., 2005) are in strong agreement with these interpretations (Choi and Gruetter, 2004).

The important brain functions underscored from the study of aralar-knockout mice may be relevant for two human diseases of the nervous system. A strong linkage and association of the gene for aralar, *SLC25A12*, with autism has been reported (Ramoz et al., 2004; Segurado et al., 2005; but see Blasi et al.,

2005). Two polymorphisms that correspond to single-nucleotide polymorphisms (SNPs) in flanking intronic sequences of exons 4 and 16 of *SLC25A12* were associated with autism, although the functional relevance of these SNPs is as yet unknown (Ramoz et al., 2004; Segurado et al., 2005). Expression of a number of genes is regulated by *cis*-acting elements, and inherited variation in gene expression may contribute to disease (Bray et al., 2003). Altered regulation of gene expression without variation in its coding sequence could affect cellular functions when levels of the gene product are limiting, as appears to be the case for aralar, as overexpression of this protein in β cells increases MAS function (Rubi et al., 2004).

Impaired aralar activity could also contribute to the pathology of Mohr-Tranebjaerg syndrome (MTS/DFN-1, deafness/dystonia syndrome), which is caused by mutations in the human Tim8p locus (*DDP1/TIMM8a* locus; Koehler et al., 1999; Roesch et al., 2002). The Tim8p mutations that MTS/DFN-1 patients appear to have impaired import of aralar, as a lymphoblast cell line derived from an MTS patient showed decreased mitochondrial NADH levels because of defects in MAS activity (Roesch et al., 2004).

Thus, genetic variations that change the expression of aralar or impaired import of aralar in mitochondria could lead to changes in MAS function, respiration on glutamate and malate, aspartate synthesis by neuronal mitochondria, and N-acetylaspartate production, which could be related to these CNS diseases. Some studies have reported decreased NAA in different brain regions of autistic patients (Otsuka et al., 1999; Friedman et al., 2003), but others have not (Friedman et al., 2006; Zeegers et al., 2006).

ROLE OF ARALAR IN CA^{2+} SIGNALING IN NEURONAL MITOCHONDRIA

Cytosolic NADH produced during glycolysis needs to be reoxidized through the MAS or another NADH shuttle in order to avoid a block in glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase (Fig. 2). MAS activity results in the production of mitochondrial NADH. A Ca^{2+} signal arising in the cytosol is thought to be transduced to mitochondria through the passage of Ca^{2+} across the inner mitochondrial membrane along the Ca^{2+} uniporter (CaU), a much looked-at (Villa et al., 1998; Carafoli, 2003; Saris and Carafoli, 2005) but still unidentified transporter protein. Then the Ca^{2+} signal arising in mitochondria, or the Ca^{2+} mark, a term applied to a miniature Ca^{2+} signal from a single mitochondrion (Hajnoczky et al., 2002; Pacher et al., 2002), activates three Ca^{2+}-dependent dehydrogenases in the mitochondrial matrix (pyruvate, α-ketoglutarate, and isocitrate dehydrogenase), resulting in increased mitochondrial NADH (McCormack, 1985, 1990; Nichols and Denton, 1995). Ca^{2+} acting on the external side of the inner mitochondrial membrane may activate the aralar–MAS pathway, resulting in an increase in mitochondrial NADH independent of CaU activity (Fig. 2).

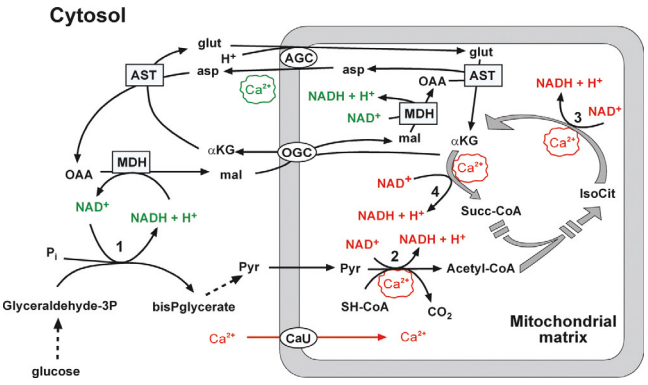

Fig. 2. Ca^{2+} activation of the aspartate-glutamate carrier (AGC)–malate–aspartate NADH shuttle pathway (aralar–MAS) and the calcium uniporter–mitochondrial dehydrogenases pathway (CaU–mitDH) by calcium. The MAS is made up of four enzymes, mitochondrial and cytosolic aspartate aminotransferases (AST) and malate dehydrogenases (MDH), and two mitochondrial carriers, the AGC and the α-ketoglutarate–malate carrier (OGC). MAS activity results in the transfer to the mitochondria of reducing equivalents generated with the reaction of glyceraldehyde-3-phosphate dehydrogenase (1). Extramitochondrial Ca^{2+} can activate the aralar–MAS pathway, resulting in increased mitochondrial NADH, in green. Mitochondrial matrix Ca^{2+}, taken up through the Ca^{2+} uniporter (CaU), activates pyruvate dehydrogenase, PDH (2), isocitrate dehydrogenase, IDH (3), and α-ketoglutarate dehydrogenase, α-KGDH (4), generating intramitochondrial NADH, in red (asp, aspartate; glut, glutamate; IsoCit, isocitrate; α-KG, α-ketoglutarate; mal, malate; OAA, oxaloacetic acid; Pyr, pyruvate; Succ-CoA, succinyl-CoA).

MAS activity is stimulated by calcium from the external side of mitochondria (Palmieri et al., 2001).This allows the study of the kinetics of Ca^{2+} activation of the MAS in the presence of ruthenium red (RR) to block Ca^{2+} uptake in mitochondria through the CaU. The $S_{0.5}$ for Ca^{2+} stimulation of the in brain mitochondrial MAS, which contains aralar as its only AGC isoform, was around 300 nM (Pardo et al., 2006), and similar values were obtained in mitochondria from tissues expressing aralar as the only AGC isoform (Contreras et al., 2007). This value is lower than those at which the CaU is active (2–20 μM; Gunter et al., 2000; Moreau et al., 2006), which could allow activation of the aralar–MAS pathway with Ca^{2+} signals below the threshold of the CaU.

Pardo et al. (2006) employed primary neuronal cultures from control and aralar-deficient mice and NAD(P)H imaging with two-photon excitation microscopy to explore this issue. Lactate utilization involves NADH shuttle activity and is important in neurons as part of the astrocyte–neuron lactate shuttle (Pellerin and Magistretti, 1994). Lactate is produced by astrocytes and taken up by neurons that use it as an oxidizable substrate, especially during high activity (Pellerin et al., 1998; Kasischke et al., 2004; Pellerin and Magistretti, 2004). The addition of 20 mM lactate caused an increase

in cytosolic and mitochondrial NAD(P)H and a substantial transfer of reducing equivalents to mitochondria in the control neurons but not in the aralar-deficient neurons (Pardo et al., 2006), indicating that the MAS is the main NADH shuttle in neurons.

To study the effect of small cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) signals on MAS activity, neurons were incubated in Ca^{2+}-free medium plus 100 μM EGTA and exposed to lactate together with 100 μM ATP or different Ca^{2+} mobilizing agonists (Pardo et al., 2006). In control and aralar-deficient neurons, ATP-induced $[Ca^{2+}]_i$ transients were small, with departures no more than 100 nM from resting values, and they were not accompanied by any detectable increase in mitochondrial free calcium ($[Ca^{2+}]_{mit}$), as measured with rhod-2. However, this small Ca^{2+} signal resulted in remarkable potentiation of a lactate-dependent increase in mitochondrial NAD(P)H fluorescence in control but not in aralar-deficient neurons. Other Ca^{2+}-mobilizing agonists such as carbachol and thapsigargin also significantly potentiated the response to lactate in control neurons (Pardo et al., 2006).

Because lactate-derived pyruvate contributes to NAD(P)H generation in mitochondria, Pardo et al. (2006) also tested whether the effects of ATP involve Ca^{2+}-stimulated pyruvate metabolism rather than Ca^{2+}-stimulated MAS activity. Remarkably, the small $[Ca^{2+}]_i$ signals triggered by ATP had no effect on the increase in mitochondrial NAD(P)H induced by pyruvate. Therefore, the results clearly showed that the neuronal aralar–MAS pathway is selectively activated by small Ca^{2+} signals that are below the threshold for Ca^{2+} uniporter activation.

Synaptosomal mitochondria start taking up Ca^{2+} at a global $[Ca^{2+}]_i$ of about 350–400 nM (Martínez-Serrano and Satrústegui, 1992), whereas the $S_{0.5}$ for Ca^{2+} activation of the aralar–MAS pathway is about 300 nM (Pardo et al., 2006). This suggests that Ca^{2+} signaling through the aralar–MAS pathway in neuronal mitochondria would be significantly below these Ca^{2+} concentrations, which is exactly what Pardo et al. (2006) found. On the other hand, finding that mitochondria respond to $[Ca^{2+}]_i$ signals with a substantial increase in NAD(P)H through a Ca^{2+} uniporter-independent pathway was new and surprising, specially because the magnitude of the response via the aralar–MAS pathway was very large in neurons, not far from that elicited by the activity of the Ca^{2+} uniporter–mitochondrial dehydrogenases pathway.

The effects of large $[Ca^{2+}]_i$ signals on the aralar–MAS pathway were studied using high K^+ depolarization to activate voltage-operated Ca^{2+} channels (VOCC) in the presence of millimolar external Ca^{2+} concentrations (Pardo et al., 2006). There was no difference in the increases in $[Ca^{2+}]_i$, and $[Ca^{2+}]_{mit}$ obtained after the addition of lactate plus KCl in control and aralar-deficient neurons, which also did not differ in the lactate-induced increase in the mit/cyt NAD(P)H fluorescence ratio potentiated by the large $[Ca^{2+}]_i$, signals (Pardo et al., 2006). As the increase in mitochondrial NAD(P)H obtained with high K^+ was expected to result from the additive effects of Ca^{2+} activation on MAS activity (aralar–MAS pathway) and Ca^{2+} activation of mitochondrial dehydrogenases (CaU–mitDH pathway), the lack of difference between wild-type and aralar-deficient neurons indicated that MAS activity contributes very little to the increase in mitochondrial NAD(P)H with high K^+ depolarization, suggesting that MAS activity may be inhibited in conditions that allow mitochondrial Ca^{2+} uptake.

Studies of MAS activity in isolated mitochondria in the presence or the absence of 200 nM RR showed that Ca^{2+} activation of the MAS was diminished if no RR was present (Pardo et al., 2006). The block in Ca^{2+} activation of the MAS observed when Ca^{2+} enters mitochondria is probably a result of competition for substrate between α-ketoglutarate dehydrogenase (α-KGDH) and the α-ketoglutarate–malate transporter (OGC) of the MAS. The two reactions compete for α-ketoglutarate (α-KG) by their apparent K_m values. The K_m for α-KG of the OGC on the matrix side of the carrier is 1.5 mM (Sluse et al., 1973) and that of α-KGDH is around 0.2 mM (Rutter and Denton, 1988). Activation by Ca^{2+} of α-KGDH increases its affinity for α-KG (Rutter and Denton, 1988; Nichols and Denton, 1995), resulting in a decrease in α-KG level, which may cause a reduction of OGC activity and inhibition of MAS activity, as observed in the heart (O'Donnell et al., 1998, 2004). Another factor that may blunt MAS activation when all mitochondrial dehydrogenases are activated by Ca^{2+} is the thermodynamic constraint of proton gradient formation. If the proton electrochemical gradient obtained in those conditions is close to maximal and cannot be increased, it would limit the entry of reducing equivalents into mitochondria through the MAS. This situation was described in β cells incubated with different fuel secretagogues that bypass glucose metabolism, each of them setting a maximal hyperpolarization of the mitochondrial membrane that correlated with insulin secretion (Antinozzi et al., 2002).

The results obtained in neurons suggest that large Ca^{2+} signals generate increased NAD(P)H mainly from activation of Krebs cycle dehydrogenases rather than from MAS activity. Inhibition of the MAS probably disappears when the activation by Ca^{2+} of mitochondrial dehydrogenases comes to an end and α-KG and the mitochondrial proton electrochemical gradient return to control values. Thus, it is possible that after the decay of $[Ca^{2+}]_{mit}$ transients, MAS activity could prolong the increase in mitochondrial NAD(P)H induced by high $[Ca^{2+}]_i$ signals, and thus contribute to ATP synthesis and recovery of the resting state, conditions that rely on neuronal lactate utilization (Pellerin and Magistretti, 1994, 2004).

In sum, the aralar–MAS pathway provides a new mechanism for Ca^{2+} signaling in mitochondria. Together with the Ca^{2+} uniporter these systems make up a mitochondrial decoding tool kit by which each cytosolic

Ca^{2+} signal translates into a physiological response of mitochondria: an increased supply of substrate (NADH) to the electron transport chain. The decoding devices differ in their sensitivity to Ca^{2+}. The aralar–MAS pathway is activated by Ca^{2+} at concentrations below those at which the Ca^{2+} uniporter operates. For unitary brief and highly localized Ca^{2+} signals, the aralar–MAS pathway could respond both earlier than the Ca^{2+} uniporter and after the Ca^{2+} mark has ended, even in conditions in which the Ca^{2+} uniporter does not respond. In serving as the advance guard and the rear guard of the mitochondrial response to the Ca^{2+} signal, the aralar–MAS pathway may prime mitochondria to respond to Ca^{2+} entry by prolonging mitochondrial energization beyond the duration of the individual Ca^{2+} mark through an increase in the reduction state of mitochondrial NADH and a decrease in the redox state of cytosolic NADH. As shown by Cerdán et al. (2006), lactate utilization in neurons imposes a redox block on glucose utilization by its effects on the cytosolic lactate/pyruvate and NADH/NAD ratios. By removing this block, Ca^{2+} activation of the aralar–MAS pathway may allow for both a localized increase in glycolysis and an increased supply of NADH to mitochondria.

REFERENCES

Antinozzi PA, Ishihara H, Newgard CB, Wollheim CB. 2002. Mitochondrial metabolism sets the maximal limit of fuel-stimulated insulin secretion in a model pancreatic beta cell: a survey of four fuel secretagogues. J Biol Chem 277:11746–11755.

Aprille JR. 1993. Mechanism and regulation of the mitochondrial ATP-Mg/P(i) carrier. J Bioenerg Biomembr 25:473–481.

Balázs R. 1965. Control of glutamate oxidation in brain and liver mitochondrial systems. Biochem J 95:497–508.

Baslow MH. 2003. N-acetylaspartate in the vertebrate brain: metabolism and function. Neurochem Res 28:941–953.

Begum L, Jalil MA, Kobayashi K, Iijima M, Li MX, Yasuda T, Horiuchi M, del Arco A, Satrústegui J, Saheki T. 2002. Expression of three mitochondrial solute carriers, citrin, aralar1 and ornithine transporter, in relation to urea cycle in mice. Biochim Biophys Acta 1574:283–292.

Bhakoo KK, Craig TJ, Styles P. 2001. Developmental and regional distribution of aspartoacylase in rat brain tissue. J Neurochem 79:211–220.

Blasi F, Bacchelli E, Carone S, Toma C, Monaco AP, Bailey AJ, Maestrini E. 2005. SLC25A12 and CMYA3 gene variants are not associated with autism in the IMGSAC multiplex family sample. Eur J Hum Genet 14:123–126.

Bray NJ, Buckland PR, Owen MJ, O'Donovan MC. 2003. Cis-acting variation in the expression of a high proportion of genes in human brain. Hum Genet 113:149–153.

Burri R, Steffen C, Herschkowitz N. 1991. N-acetyl-aspartate is a major source of acetyl groups for lipid synthesis during rat brain development. Dev Neurosci. 13:403–411.

Carafoli E. 2003. Historical review: mitochondria and calcium: ups and downs of an unusual relationship. Trends Biochem Sci 28:175–181.

Cavero S, Vozza A, del Arco A, Palmieri L, Villa A, Blanco E, Runswick MJ, Walker JE, Cerdan S, Palmieri F, Satrústegui J. 2003. Identification and metabolic role of the mitochondrial aspartate-glutamate transporter in *Saccharomyces cerevisiae*. Mol Microbiol 50:1257–1269.

Cavero S, Traba J, del Arco A, Satrústegui J. 2005 The calcium dependent ATP-Mg/Pi mitochondrial carrier is a target of glucose-induced calcium signalling in *Saccharomyces cerevisiae*. Biochem J 392:537–544.

Cerdan S, Rodrigues TB, Sierra A, Benito M, Fonseca LL, Fonseca CP, García-Martín ML. 2006. The redox switch/redox coupling hypothesis. Neurochem Int 48:523–530.

Chakraborty G, Mekala P, Yahya D, Wu G, Ledeen RW. 2001. Intraneuronal N-acetylaspartate supplies acetyl groups for myelin lipid synthesis: evidence for myelin-associated aspartoacylase. J Neurochem 78:736–745.

Cheeseman AJ, Clark JB. 1988. Influence of the malate-aspartate shuttle on oxidative metabolism in synaptosomes. J Neurochem 50:1559–1565

Choi I-Y, Gruetter R. 2004. Dynamic or inert metabolism? Turnover of N-acetyl aspartate and glutathione from D-[1-^{13}C]glucose in the rat brain in vivo. J Neurochem 91:778–787.

Clark JB. 1998. N-acetylaspartate: a marker for neuronal loss or mitochondrial dysfunction. Dev Neurosci 20:271–276.

Contreras L, Gomez-Puertas P, Iijima M, Kobayashi K, Saheki T, Satrústegui J. 2007 Ca^{2+} activation kinetics of the two aspartate-glutamate mitochondrial carriers aralar and citrin: Role in heart malate-aspartate NADH shuttle. J Biol Chem 282:7098–7106.

D'Adamo AF Jr, Yatsu FM. 1966. Acetate metabolism in the nervous system. N-acetyl-L-aspartic acid and the biosynthesis of brain lipids. J Neurochem 13:961–965.

del Arco A. 2005. Novel variants of human SCaMC-3, an isoform of the ATP-Mg/P(i) mitochondrial carrier, generated by alternative splicing from 3′-flanking transposable elements. Biochem J 389:647–655.

del Arco A, Agudo M, Satrústegui J. 2000. Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitable tissues. Biochem J 345:725–732.

del Arco A, Morcillo J, Martínez-Morales JR, Galián C, Martos V, Bovolenta P, Satrústegui J. 2002. Expression of the aspartate/glutamate mitochondrial carriers aralar1 and citrin during development and in adult rat tissues. Eur J Biochem 269:3313–3320.

del Arco A, Satrústegui J. 2004. Identification of a novel human subfamily of mitochondrial carriers with calcium-binding domains. J Biol Chem 279:24701–24713.

del Arco A, Satrústegui J. 1998. Molecular cloning of Aralar, a new member of the mitochondrial carrier superfamily that binds calcium and is present in human muscle and brain. J Biol Chem 273:23327–23334.

del Arco A, Satrústegui J. 2005. New mitochondrial carriers: an overview. Cell Mol Life Sci 62:2204–2227.

Dienel GA, Cruz NF. 2006. Astrocyte activation in working brain: energy supplied by minor substrates. Neurochem Int 48:586–595.

Fiermonte G, Palmieri L, Todisco S, Agrimi G, Palmieri F, Walker JE. 2002. Identification of the mitochondrial glutamate transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. J Biol Chem 277:19289–19294.

Fiermonte G, De Leonardis F, Todisco S, Palmieri L, Lasorsa FM, and Palmieri F. 2004. Identification of the mitochondrial ATP-Mg/Pi transporter: bacterial expression, reconstitution, functional characterization and tissue distribution. J Biol Chem 279:30722–30730.

Friedman SD, Shaw DW, Artru AA, Richards TL, Gardner J, Dawson G, Posse S, Dager SR. 2003. Regional brain chemical alterations in young children with autism spectrum disorder. Neurology 60:100–107.

Friedman SD, Shaw DW, Artru AA, Dawson G, Petropoulos H, Dager SR. 2006. Gray and white matter brain chemistry in young children with autism. Arch Gen Psychiatry 63:786–794.

Fujita T, Katsukawa H, Yodoya E, Wada M, Shimada A, Okada N, Yamamoto A, Ganapathy V. 2005. Transport characteristics of N-acetyl-L-aspartate in rat astrocytes: involvement of sodium-coupled high-affinity carboxylate transporter NaC3/NaDC3-mediated transport system. J Neurochem 93:706–714.

Gunter TE, Buntinas L, Sparagna G, Eliseev R, Gunter K. 2000. Mitochondrial calcium transport: mechanisms and functions. Cell Calcium 28:285–296.

Hajnoczky G, Csordas G, Yi M. 2002. Old players in a new role: mitochondria-associated membranes, VDAC, and ryanodine receptors as contributors to calcium signal propagation from endoplasmic reticulum to the mitochondria. Cell Calcium 32:363–377.

Jalil MA, Begum L, Contreras L, Pardo B, Iijima M, Li MX, Ramos M, Marmol P, Horiuchi M, Shimotsu K, Nakagawa S, Okubo A, Sameshima M, Isashiki Y, del Arco A, Kobayashi K, Satrústegui J, Saheki T. 2005. Reduced N-acetylaspartate levels in mice lacking aralar, a brain- and muscle-type mitochondrial aspartate-glutamate carrier. J Biol Chem 280:31333–339.

Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. 2004. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. Science 305:99–103.

Kirmani BF, Jacobowitz DM, Namboodiri MA. 2003. Developmental increase of aspartoacylase in oligodendrocytes parallels CNS myelination. Brain Res Dev Brain Res 140:105–115.

Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H, Crackower MA, Kondo I, Tsui LC, Scherer SW, Saheki T. 1999. The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. Nat Genet 22:159–163.

Koehler CM, Leuenberger D, Merchant S, Renold A, Junne T, Schatz G. 1999. Human deafness dystonia syndrome is a mitochondrial disease. Proc Natl Acad Sci U S A 96:2141–2146.

Kunnecke B, Cerdan S, Seelig J. 1993. Cerebral metabolism of [1,2-13C2]glucose and [U-13C4]3-hydroxybutyrate in rat brain as detected by 13C NMR spectroscopy. NMR Biomed. 6:264–277.

LaNoue KF, Schoolwerth AC. 1979. Metabolite transport in mitochondria. Annu Rev Biochem 48:871–922.

LaNoue KF, Berkich DA, Conway M, Barber AJ, Hu L-Y, Taylor C, Hutson S. 2001. Role of specific aminotransferases in de novo glutamate synthesis and redox shuttling in the retina. J Neurosci Res 66:914–922.

Lu ZH, Chakraborty G, Ledeen RW, Yahya D, Wu G. 2004. N-Acetylaspartate synthase is bimodally expressed in microsomes and mitochondria of brain. Brain Res Mol Brain Res 122:71–78.

Madhavarao CN, Chinopoulos C, Chandrasekaran K, Namboodiri MA. 2003. Characterization of the N-acetylaspartate biosynthetic enzyme from rat brain. J Neurochem 86:824–835.

Madhavarao CN, Arun P, Moffett JR, Szucs S, Surendran S, Matalon R, Garbern J, Hristova D, Johnson A, Jiang W, Namboodiri MA. 2005. Defective N-acetylaspartate catabolism reduces brain acetate levels and myelin lipid synthesis in Canavan's disease. Proc Natl Acad Sci U S A 102:5221–5226.

Martínez-Serrano A, Satrústegui J. 1992. Regulation of cytosolic free calcium concentration by intrasynaptic mitochondria. Mol Biol Cell 3:235–248.

Matalon R, Rady PL, Platt KA, Skinner HB, Quast MJ, Campbell GA, Matalon K, Ceci JD, Tyring SK, Nehls M, Surendran S, Wei J, Ezell EL, Szucs S. 2000. Knock-out mouse for Canavan disease: a model for gene transfer to the central nervous system. J Gene Med 2:165–175.

McCormack JG. 1985. Characterization of the effects of c on the intramitochondrial Ca^{2+}-sensitive enzymes from rat liver and within intact rat liver mitochondria. Biochem J 231:581–595.

McCormack JG, Halestrap AP, Denton RM. 1990. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol Rev 70:391–425.

McKenna MC, Tildon JT, Stevenson JH, Boatright R, Huang S. 1993. Regulation of energy metabolism in synaptic terminals and cultured rat brain astrocytes: differences revealed using aminooxyacetate. Dev Neurosci 15:320–329.

McKenna MC, Waagepetersen HS, Schousboe A, Sonnewald U. 2006. Neuronal and astrocytic shuttle mechanisms for cytosolic-mitochondrial transfer of reducing equivalents: Current evidence and pharmacological tools. Biochem Pharmacol 71:399–407.

Mehta V, Namboodiri MA. 1995. N-acetylaspartate as an acetyl source in the nervous system. Brain Res Mol Brain Res 31:151–157.

Moreau B, Nelson C, Parekh AB. 2006. Biphasic regulation of mitochondrial Ca^{2+} uptake by cytosolic Ca^{2+} concentration. Curr Biol 16:1672–1677.

Namboodiri ,AMA, Peethambaran A, Mathew R, Sambhu PA, Hershfield J, Moffett JR, Madhavarao CN. 2006. Canavan disease and the role of N-acetylaspartate in myelin synthesis. Mol Cell Endocrinol 252:216–223.

Nichols BJ, Denton RM. 1995. Towards the molecular basis for the regulation of mitochondrial dehydrogenases by calcium ions. Mol Cell Biochem 149–150:203–212.

O'Donnell JM, Doumen C, LaNoue KF, White LT, Yu X, Alpert NM, Lewandowski ED. 1998. Dehydrogenase regulation of metabolite oxidation and efflux from mitochondria in intact hearts. Am J Physiol 274:H467–H476.

O'Donnell JM, Kudej RK, LaNoue KF, Vatner SF, Lewandowski ED. 2004. Limited transfer of cytosolic NADH into mitochondria at high cardiac workload. Am J Physiol Heart Circ Physiol 286:H2237–H2242.

Otsuka H, Harada M, Mori K, Hisaoka S, Nishitani H. 1999. Brain metabolites in the hippocampus-amygdala region and cerebellum in autism: an 1H-MR spectroscopy study. Neuroradiology 41:517–519.

Pacher P, Thomas AP, Hajnoczky G. 2002. Ca^{2+} marks: miniature calcium signals in single mitochondria driven by ryanodine receptors. Proc Natl Acad Sci U S A 99:2380–2385.

Palmieri L, Pardo B, Lasorsa FM, del Arco A, Kobayashi K, Iijima M, Runswick MJ, Walker JE, Saheki T, Satrústegui J, Palmieri F. 2001. Citrin and aralar1 are Ca^{2+}-stimulated aspartate/glutamate transporters in mitochondria. Embo J 20:5060–5069.

Pan JW, Takahashi K. 2005. Interdependence of N-acetyl aspartate and high-energy phosphates in healthy human brain. Ann Neurol 57:92–97.

Pardo B, Contreras L, Serrano A, Ramos M, Kobayashi K, Iijima M, Saheki T, Satrústegui J. 2006. Essential role of aralar in the transduction of small Ca^{2+} signals to neuronal mitochondria. J Biol Chem 281: 1039–1047.

Patel TB, Clark JB. 1979. Synthesis of N-acetyl-L-aspartate by rat brain mitochondria and its involvement in mitochondrial/cytosolic carbon transport. Biochem J 184:539–546.

Pebay-Peyroula E, Brandolin G. 2004. Nucleotide exchange in mitochondria: insight at a molecular level. Curr Opin Struct Biol 14:420–425.

Pellerin L, Magistretti PJ. 1994. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A 91:10625–10629.

Pellerin L, Magistretti PJ. 2004. Neuroscience. Let there be (NADH) light. Science 305:50–52.

Pellerin L, Pellegri G, Bittar PG, Charnay Y, Bouras C, Martin JL, Stella N, Magistretti PJ. 1998. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. Dev Neurosci 20:291–299.

Price MT, Pusateri ME, Crow SE, Buchsbaum S, Olney JW, Lowry OH. 1984. Uptake of exogenous aspartate into circumventricular organs but not other regions of adult mouse brain. J Neurochem 42: 740–744.

Ramos M, del Arco A, Pardo B, Martinez-Serrano A, Martinez-Morales JR, Kobayashi K, Yasuda T, Bogonez E, Bovolenta P, Saheki T, Satrústegui J. 2003. Developmental changes in the Ca^{2+}-regulated mitochondrial aspartate-glutamate carrier aralar1 in brain and prominent expression in the spinal cord. Dev Brain Res 143:33–46.

Ramoz N, Reichert JG, Smith CJ, Silverman JM, Bespalova IN, Davis KL, Buxbaum JD. 2004. Linkage and association of the mitochondrial

aspartate/glutamate carrier SLC25A12 gene with autism. Am J Psychiatry 161:662–669.

Roesch K, Curran SP, Tranebjaerg L, Koehler CM. 2002. Human deafness dystonia syndrome is caused by a defect in assembly of the DDP1/TIMM8a-TIMM13 complex. Hum Mol Genet 11:477–486.

Roesch K, Hynds PJ, Varga R, Tranebjaerg L, Koehler CM. 2004. The calcium-binding aspartate/glutamate carriers, citrin and aralar1, are new substrates for the DDP1/TIMM8a-TIMM13 complex. Hum Mol Genet 13:2101–2111.

Rubi B, del Arco A, Bartley C, Satrústegui J, Maechler P. 2004. The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. J Biol Chem 279:55659–55666.

Rutter GA, Denton RM. 1988. Regulation of NAD^+-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase by Ca^{2+} ions within toluene-permeabilized rat heart mitochondria. Interactions with regulation by adenine nucleotides and $NADH/NAD^+$ ratios. Biochem J 252: 181–189.

Sager TN, Thomsen C, Valsborg JS, Laursen H, Hansen AJ. 1999. Astroglia contain a specific transport mechanism for N-acetyl-aspartate. J Neurochem 73:807–811.

Sambhu PA, Madhavarao CN, Namboodiri MA. 2005. Characterization of aspartate-N-acetyltransferase from bovine brain. Abstr Soc Neurosci 18813.

Sanz R, del Arco A, Ayuso C, Ramos C, Satrústegui J. 2000. Calcium-binding mitochondrial carrier Aralar1 gene (SLC25A12) to human chromosome band 2q31 by in situ hybridization. Cytogenet Cell Genet 89:143–144.

Saris NE, Carafoli E. 2005. A historical review of cellular calcium handling, with emphasis on mitochondria. Biochemistry (Mosc) 70:187–194.

Satrústegui J, Pardo B, del Arco A. 2007. Mitochondrial transporters as novel targets for intracellular calcium signaling. Physiol Rev 87:29–67.

Segurado R, Conroy J, Meally E, Fitzgerald M, Gill M, Gallagher L. 2005. Confirmation of association between autism and the mitochondrial aspartate/glutamate carrier *SLC25A12* gene on chromosome 2q31. Am J Psychiatry 162:2182–2184.

Shen J. 2005. In vivo carbon-13 magnetization transfer effect. Detection of aspartate aminotransferase reaction. Magn Reson Med 54:1321–1326.

Sinasac DS, Crackower MA, Lee JR, Kobayashi K, Saheki T, Scherer SW, Tsui LC. 1999. Genomic structure of the adult-onset type II citrullinemia gene, SLC25A13, and cloning and expression of its mouse homologue. Genomics 62:289–292.

Sluse FE, Goffart G, Liebecq C. 1973. Mechanism of the exchanges catalysed by the oxoglutarate translocator of rat-heart mitochondria. Kinetics of the external-product inhibition. Eur J Biochem 32:283–291.

Tsai G, Coyle JT. 1995. N-acetylaspartate in neuropsychiatric disorders. Progr Neurobiol 46:531–540.

Tyson RL.Sutherland GR. 1998. Labeling of N-acetylaspartate and N-acetylaspartylglutamate in rat neocortex, hippocampus and cerebellum from [1-13C]glucose. Neurosci Lett 251:181–184.

Urenjak J, Williams SR, Gadian DG, Noble M. 1992. Specific expression of N-acetylaspartate in neurons, oligodendrocyte-type-2 astrocyte progenitors, and immature oligodendrocytes in vitro. J Neurochem 59: 55–61.

Urenjak J, Williams SR, Gadian DG, Noble M. 1993. Proton nuclear magnetic resonance spectroscopy unambiguously identifies different neural cell types. J Neurosci 13:981–989.

Villa A, García-Simón MI, Blanco P, Sesé B, Bogónez E, Satrústegui J. 1998. Affinity chromatography purification of mitochondrial inner membrane proteins with calcium transport activity. Biochim Biophys Acta 1373:347–359.

Zeegers M, van der Grond J, van Daalen E, Buitelaar J, van Engeland H. 2006. Proton magnetic resonance spectroscopy in developmentally delayed young boys with or without autism. J Neural Transm 114:289–295.

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