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Commentary

Neuronal and astrocytic shuttle mechanisms for cytosolic-mitochondrial transfer of reducing equivalents: Current evidence and pharmacological tools☆

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ABSTRACT

The malate–aspartate shuttle and the glycerol phosphate shuttle act to transfer reducing equivalents from NADH in the cytosol to the mitochondria since the inner mitochondrial membrane is impermeable to NADH and NAD^+. This transfer of reducing equivalents is essential for maintaining a favorable NAD^+/NADH ratio required for the oxidative metabolism of glucose and synthesis of neurotransmitters in brain. There is evidence that both the malate–aspartate shuttle and glycerol phosphate shuttle function in brain; however, there is controversy about the relative importance and cellular localization of these shuttles. The malate–aspartate shuttle is considered the most important shuttle in brain. It is particularly important in neurons and may be extremely low, or even non-existent in brain astrocytes. Several studies provide evidence of glycerol phosphate shuttle activity in brain cells; however, the activity of this shuttle in brain has been questioned. A number of pharmacological tools, including aminooxyacetic acid, β-methyleneaspartate, phenylsuccinate, and 3-nitropropionic acid, have been used to inhibit the four enzymes and two carrier proteins that participate in the malate–aspartate shuttle. Although no drugs completely inhibit the glycerol phosphate shuttle, evidence for the existence of this shuttle is provided by studies using drugs to inhibit the malate–aspartate shuttle. This report evaluates the evidence for each shuttle in brain cells and the drugs that can be used as pharmacological tools to study these shuttles.

☆ The authors regret that many excellent papers dealing with the malate–aspartate shuttle and glycerol 3-phosphate dehydrogenase could not be included due to the limitation on the number of references.

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1. Introduction

Oxidative metabolism of pyruvate derived from glucose, the primary energy substrate for brain, is essential for brain function. For metabolism to continue, the NADH in the cytosol must be reoxidized to NAD^+. If this does not occur, the NADH/NAD^+ ratio increases and pyruvate is converted to lactate in order to reoxidize NADH to NAD^+. Such conversion of pyruvate to lactate prevents the oxidative metabolism of pyruvate, derived from glucose, via the tricarboxylic acid (TCA) cycle, the pathway that provides most of the energy from glucose. Since the inner mitochondrial membrane is impermeable to NADH and NAD^+, two shuttles act to transfer reducing equivalents from NADH in the cytosol to the mitochondria in brain, the malate–aspartate shuttle and the glycerol 3-phosphate shuttle [1]. The malate–aspartate shuttle is particularly important in neurons and is considered the most important shuttle in brain [2–4]. A recent study by Ramos et al. [5] provides evidence that the activity of the malate–aspartate shuttle may be extremely low, or even non-existent in brain astrocytes. It should, however, be noted that although it is assumed that the malate–aspartate shuttle is the predominant mechanism for shuttling reducing equivalents from the cytosol to the mitochondrial matrix in the brain [6], other shuttle mechanisms may operate. Although the enzymes for the glycerol 3-phosphate shuttle are also present, there is controversy about the importance of this shuttle in brain [2,7]. In this report, we evaluate the evidence for each shuttle in brain cells and the drugs that can be used as pharmacological tools to study these shuttles.

2. The shuttle mechanisms and the controversy about cell types

2.1. *The malate–aspartate shuttle*

Several proteins participate in the malate–aspartate shuttle including the aspartate–glutamate carrier, the malate–α-ketoglutarate carrier and both the cytosolic and mitochondrial forms of malate dehydrogenase (MDH) and aspartate aminotransferase (Fig. 1). This shuttle acts to transfer electrons from NADH to oxaloacetate in the cytosol forming malate, which enters the mitochondria in exchange for α-ketoglutarate via the malate–α-ketoglutarate carrier [1]. The TCA cycle enzyme mitochondrial malate dehydrogenase (mMDH) converts malate to oxaloacetate, transferring the reducing equivalents to NAD^+, forming NADH. Oxaloacetate is subsequently transaminated to aspartate via mitochondrial aspartate aminotransferase in conjunction with the conversion of glutamate to α-ketoglutarate. Aspartate leaves the mitochondria via the aspartate–glutamate carrier in exchange for cytosolic glutamate, which enters the mitochondria with a proton. To complete the shuttle cytosolic aspartate aminotransferase converts the aspartate to oxaloacetate, simultaneously forming glutamate from α-ketoglutarate. Although the malate–aspartate shuttle is reversible,

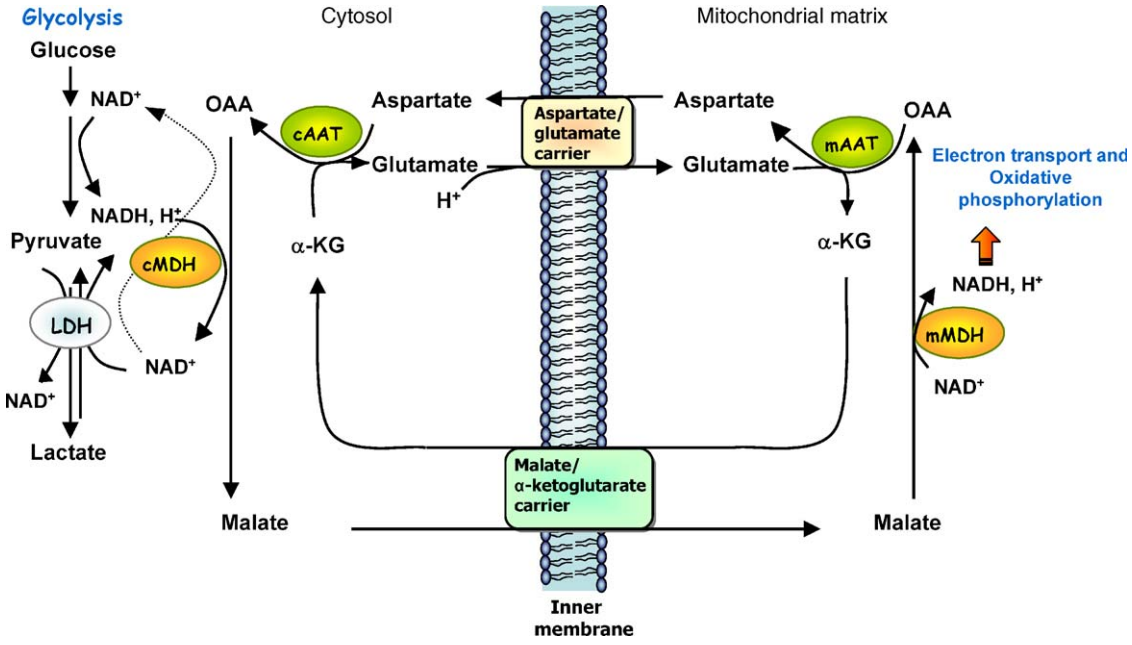

Fig. 1 – The malate–aspartate shuttle for transferring reducing equivalents from the cytosol to the mitochondria. Electrons from glycolysis or from oxidation of lactate to pyruvate are transferred from NADH, H^+ as oxaloacetate (OAA) is converted to malate by cytosolic malate dehydrogenase (cMDH). Malate enters the mitochondrial matrix via the malate–α-ketoglutarate carrier in exchange for α-ketoglutarate. Electrons are transferred to the electron transport chain as malate is oxidized to OAA by mitochondrial malate dehydrogenase (mMDH). OAA is subsequently converted to aspartate by transamination with glutamate via mitochondrial aspartate aminotransferase (mAAT). The aspartate exits the mitochondria via the aspartate–glutamate carrier (AGC1, aralar1) in an electrogenic exchange for glutamate and a proton. In the cytosol, aspartate is converted to OAA by transamination with α-ketoglutarate via cytosolic aspartate aminotransferase (cAAT) completing the shuttle. Abbreviation: LDH, lactate dehydrogenase; α-KG, α-ketoglutarate.

the electrogenic exchange of aspartate for glutamate and a proton via the aspartate–glutamate carrier is irreversible and favors efflux of aspartate from and entry of glutamate into mitochondria [8]. This exchange is stimulated by Ca^{2+} binding to a site on the outer side of the inner mitochondrial membrane. The Ca^{2+}-sensitive aspartate–glutamate carrier aralar1 (AGC1) is found in skeletal muscle and brain, and citrin (AGC2) is found primarily in liver and kidney [9]. Citrin is also found in primary cultures of mixed glial cells, but has not been found in brain tissue [5]. AGC1 is highly enriched on neuronal mitochondria; considerably lower levels of AGC1 and malate–aspartate shuttle activity are present in astrocytes [5]. Exchange of aspartate for glutamate via AGC1 appears to be the overall rate-limiting step of the shuttle [9]. Aspartate aminotransferase is the limiting enzyme of the shuttle since the activity is several-fold lower than MDH activity [2]. Activity of the malate–aspartate shuttle increases during development in parallel with synaptogenesis, consistent with the high activity and importance of this shuttle in neurons and synaptic terminals.

2.2. *The glycerol 3-phosphate shuttle*

The glycerol 3-phosphate shuttle also functions to transfer electrons from cytosolic NADH into the mitochondria (Fig. 2). However, unlike the malate–aspartate shuttle, the glycerol 3-phosphase shuttle is not reversible. The reducing equivalents from NADH are transferred to dihydroxyacetone phosphate via the enzyme glycerol 3-phosphate dehydrogenase to form glycerol 3-phosphate [1]. Glycerol 3-phosphate is converted to dihydroxyacetone phosphate on the outer surface of the inner mitochondrial membrane by mitochondrial glycerol 3-phosphate dehydrogenase, and the reducing equivalents are transferred to FAD, forming FADH, within the mitochondria. The reducing equivalents are subsequently transferred to coenzyme Q in the respiratory chain. By using FAD, this shuttle allows for transfer of reducing equivalents into the mitochondria against an NADH concentration gradient. The glycerol 3-phosphate shuttle yields less energy since the electrons are transferred to FAD, rather than NAD^+ [1]. The glycerol 3-phosphate shuttle is activated by Ca^{2+}, which decreases the K_m of the enzyme for glycerol 3-phosphate [10].

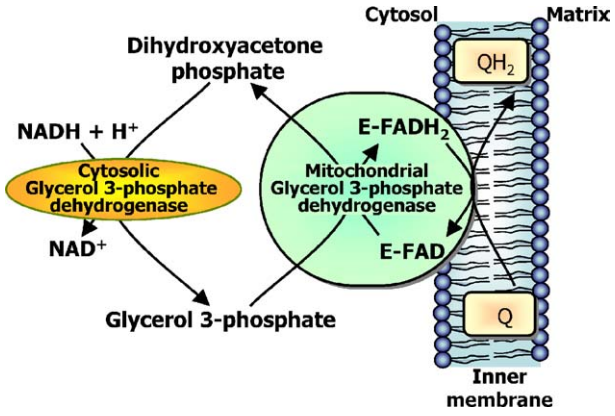

Fig. 2 – The glycerol 3-phosphate shuttle for transferring reducing equivalents from the cytosol to the mitochondria. Electrons are transferred from NADH when dihydroxyacetone phosphate is reduced to glycerol 3-phosphate. Glycerol 3-phosphate is reoxidized to dihydroxyacetone phosphate by mitochondrial glycerol 3-phosphate dehydrogenase that is bound to an FAD prosthetic group on the outer side of the inner mitochondrial membrane and electrons are transferred to coenzyme Q (Q) and subsequently enter the electron transport chain. Less energy is produced when electrons transferred into the mitochondria via the glycerol 3-phosphate shuttle enter the electron transport chain since FAD is the acceptor rather than NAD.

Cytosolic glycerol 3-phosphate dehydrogenase is highly enriched in cultured oligodendroglial cells and in white matter in brain [11]. In studies using activity-based staining, Nguyen et al. [7] reported that mitochondrial glycerol 3-phosphate dehydrogenase is localized in neurons. These authors suggested that the glycerol 3-phosphate shuttle ''is of little importance in brain'' since there is evidence of selective expression of the cytosolic form of glycerol 3-phosphate dehydrogenase in glia and the mitochondrial form in neurons [7]. However, studies with cultured astrocytes and cerebellar granule cells provide evidence of glycerol 3-phosphate shuttle activity in these cells [12–15]. Glucose and lactate homeostasis, which depends on the ability to regenerate NAD^+ in the cytosol, was found to be only marginally affected by inhibiting the malate–aspartate shuttle in astrocytes, suggesting that the glycerol-3-P dehydrogenase shuttle was active in these cells [15]. In addition, studies on the capacity of mitochondria in cortical neurons to reduce MTT (3(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazoliun bromide) showed that the reduction capacity was supported by substrates for the glycerol 3-phosphate shuttle or substrates for the malate–aspartate shuttle [5], suggesting that both the shuttles are present in primary cultures of neurons [5]. Further studies using double-labeled immunohistochemistry are needed to clarify the localization of the glycerol 3-phosphate dehydrogenase isoforms, which although highly enriched in oligodendroglial cells and Bergmann glia, appear to be present in other brain cells [12–15].

3. Use of drugs as pharmacological tools to study the shuttle mechanisms

There are no drugs that specifically inhibit the glycerol 3-phosphate shuttle. However, propyl gallate and other polyhydroxybenzoates inhibit the L-ascorbic acid and iron stimulated activity of glycerol 3-phosphate dehydrogenase from pig brain [16]. The lack of a pharmacological tool to totally inhibit the glycerol 3-phosphate shuttle in brain makes it difficult to study the importance of this shuttle in brain cells. However, continued oxidative metabolism of glucose in the presence of drugs that inhibit the malate–aspartate shuttle provides evidence of glycerol 3-phosphate shuttle activity in brain cells [13,15]. A variety of drugs have been used to study the functional importance of the malate–aspartate shuttle in the brain and brain cells. Generally, these drugs act on the enzymes and transporters that are parts of the shuttle mechanism. However, other mechanisms of action of these drugs may be apparent such as control of the availability of

substrates for the shuttle. One major problem with some of these drugs is that often other enzymes or transporters involved in metabolic pathways distinct from the malate–aspartate shuttle function are affected and thus unwanted side effects are encountered [17–19].

3.1. *Aminooxyacetic acid (AOAA) is often used, but is not specific for the malate–aspartate shuttle*

One of the most frequently used drugs is aminooxyacetic acid (AOAA), which is a carbonyl-trapping agent. This means that enzymes dependent on pyridoxal phosphate as a co-enzyme may be inhibited, an action which is related to the concentration of AOAA. AOAA is only moderately potent as an inhibitor of aspartate aminotransferase [2,20]; therefore, in order to inhibit the malate–aspartate shuttle by blocking aspartate aminotransferase an AOAA concentration of 1–5 mM has been used [20]. Thus, several other aminotransferases are likely to be inhibited and in particular the GABA metabolizing enzymes glutamate decarboxylase and GABA-aminotransferase are extremely sensitive to AOAA exhibiting K_i values of 0.5 and 0.05 μM, respectively [17,18]. This means that it is not possible to use this pharmacological tool to investigate the functional role of the malate–aspartate shuttle in brain tissue or cells if at the same time GABAergic neurotransmission should remain intact.

3.2. *β-Methyleneaspartate (BMA) inhibits aspartate aminotransferase and the malate–aspartate shuttle, but is not specific due to inhibition of glutamate uptake*

The development of BMA, as an irreversible, active site directed inhibitor of aspartate aminotransferase [21] seemed promising with regard to selectively inhibiting the malate–aspartate shuttle [2,3]. Thus, this compound could be used to demonstrate a link between the activity of the malate–aspartate shuttle, oxygen consumption and the cytosolic NAD^+/NADH ratio in cerebral cortical slices [2]. However, BMA was shown to also inhibit alanine aminotransferase and glutamate decarboxylase although inhibition of the former enzyme could be indirectly related to the inhibitory action on aspartate aminotransferase [2]. It should also be pointed out that BMA was later shown to be a potent inhibitor of astrocytic and neuronal high affinity glutamate transport [19,22]. Actually, the potency with regard to inhibition of glutamate transport is 5–10-fold higher than that of the inhibition of aspartate aminotransferase [19,21]. Therefore, the use of BMA as a selective inhibitor of malate–aspartate shuttle activity and thus to assess the functional role of this shuttle in oxidative metabolism in the brain and isolated brain cells calls for caution. This appears to be particularly important when oxidative metabolism of glutamate is investigated [3].

3.3. *Phenylsuccinate blocks the mitochondrial malate–α-ketoglutarate carrier*

The malate–aspartate shuttle involves not only the enzymes aspartate aminotransferase and malate dehydrogenase but also the mitochondrial membrane associated carriers for malate–α-ketoglutarate exchange and for aspartate–glutamate exchange. Therefore, these carrier molecules constitute alternative targets for drugs. One such drug is phenylsuccinate, which inhibits the malate–α-ketoglutarate carrier ([23] and references therein). Phenylsuccinate has been used to demonstrate the possible involvement of the malate–aspartate shuttle in the biosynthesis of neurotransmitter glutamate from glutamine [23]. It should be noted that phenylsuccinate was more effective than AOAA in inhibiting the Ca^{2+}-dependent depolarization coupled glutamate release from glutamatergic neurons utilizing exogenous glutamine as the precursor [23]. Hence, it appears that phenylsuccinate may be a useful tool to inhibit the malate–aspartate shuttle.

3.4. *3-Nitropropionic acid (3-NPA) blocks succinate dehydrogenase and thus the malate–aspartate shuttle*

The malate–aspartate shuttle activity obviously is dependent upon the availability of oxaloacetate. In astrocytes oxaloacetate can be generated by de novo synthesis by carboxylation of pyruvate [24]. Production of oxaloacetate by this anaplerotic reaction can serve to maintain some malate–aspartate shuttle activity in the presence of 3-NPA. However, in neurons this is not possible since pyruvate carboxylase catalyzing this reaction is only present in astrocytes [24]. Although it has been suggested that carboxylation can occur via malic enzyme in neurons, this finding could not be confirmed by others ([15] and references therein). If oxaloacetate cannot be generated by anaplerosis ongoing TCA cycle activity is required for maintenance of an appropriate concentration of oxaloacetate. The TCA cycle can be blocked at the level of succinate dehydrogenase using the competitive active site directed inhibitor 3-NPA as shown by Alston et al. [25]. 3-NPA acts as an irreversible inhibitor of the TCA cycle enzyme succinate dehydrogenase (Fig. 3) [25] and a reversible inhibitor of fumarase. This will lead to impairment of malate–aspartate shuttle activity as shown by the decrease in the concentrations of glutamate and aspartate in the presence of 3-NPA in organotypic corticostriatal cultures [26]. 3-NPA also inhibits respiration since succinate dehydrogenase constitutes complex II of the mitochondrial electron transport chain.

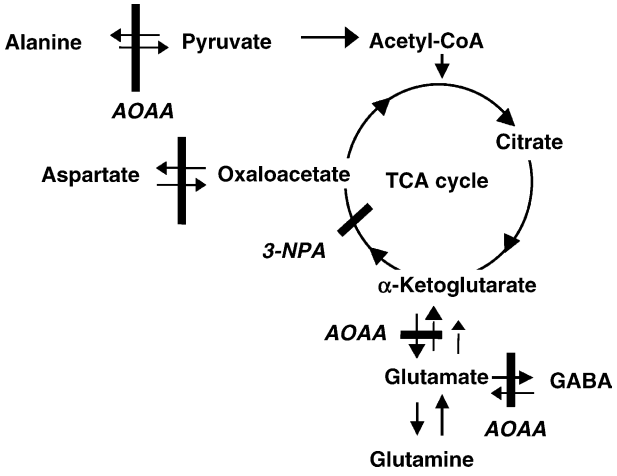

Fig. 3 – The location of blocks in metabolism by aminooxyacetate (AOAA) and 3-nitropropionic acid (3-NPA), pharmacological agents that inhibit the activity of the malate–aspartate shuttle.

4. Neurons

4.1. *The aspartate–glutamate carrier aralar1 (AGC1) is highly enriched in neurons*

The Ca^{2+}-sensitive aspartate–glutamate carrier aralar1 (AGC1) is found in skeletal muscle and brain, whereas citrin (AGC2) is found in liver and kidney ([9] and references therein). In rat and mouse brain in vivo, there is very little postnatal increase in total aralar1 levels per mitochondrion since neurons develop prenatally; whereas astrocytes, which develop postnatally, have much lower AGC1 levels than neurons [5]. Ramos et al. reported a ratio of aralar1/β-F 1 ATPase of 3 in cultured neurons, compared to a ratio of 0.8 in mixed glial cultures that contain primarily astrocytes [5]. In situ hybridization and immunocytochemistry studies show that AGC1 mRNA and protein are widely expressed in neuron-rich areas in adult mouse CNS with little labeling present in brain astrocytes [5]. AGC1 is enriched in specific neurons in the brainstem and spinal cord that are also enriched in cytochrome oxidase [5]. The levels of AGC1 increase markedly in cultured neurons during in vitro maturation, in conjunction with a prominent rise in the activity of the malate–aspartate shuttle [5].

4.2. *The malate–aspartate shuttle has an important role in neurotransmitter biosynthesis*

Numerous reports document the intimate association of the malate–aspartate shuttle and the biosynthesis of neurotransmitter glutamate [23]. Activity of this shuttle increases during development in parallel with synaptogenesis, which is consistent with the high activity and importance of this shuttle in neurons and synaptic terminals [2,4,5,13,23].

4.3. *Effect of drugs on shuttles in neurons*

Since there is so much evidence supporting the importance of the malate–aspartate shuttle in neurons and synaptosomes most of the studies have focused on this shuttle mechanism. However, a recent study by Atlante et al. [27] reported that glutamate excitotoxicity inhibited the activity of both the malate–aspartate shuttle and the glycerol 3-phosphate shuttle that were reconstituted from cerebellar granule cells. This study is important since it provides evidence of significant glycerol 3-phosphate shuttle activity in neurons and because glutamate exitotoxicity is a feature of many neurological diseases. Other studies support the concept of glycerol 3-phosphate activity in cortical neurons [5] and synaptosomes [13].

4.4. *Oxidation of glucose and lactate by neurons and/or synaptosomes is differentially affected by AOAA*

There are conflicting reports about the effect of AOAA on brain metabolism. A number of earlier studies reported that injection of AOAA into rodent brain decreased seizure activity [28]. However, more recent studies demonstrate that injection of AOAA leads to neuronal death and long lasting changes in NMDA receptor function [29]. There was no inhibition of glucose oxidation in synaptosomes incubated with 5 mM AOAA, suggesting that the malate–aspartate shuttle was not involved [13]. However, the oxidation of lactate was decreased by 50% by AOAA, demonstrating the requirement for the malate–aspartate shuttle for use of lactate for energy [13]. This profound inhibition of lactate metabolism may be due in part to the fact that malate dehydrogenase and lactate dehydrogenase are in equilibrium with the same pool of NADH [30]. This effect of AOAA on lactate metabolism may contribute to the neuronal death reported in some studies since lactate is readily oxidized by neurons for energy [31].

An increasing number of observations suggest that neurodegenerative diseases may be associated with aberrations in energy metabolism and the handling of glutamate [32]. Animal models of neurodegenerative diseases have been created employing inhibitors of mitochondrial energy metabolism. The malate–aspartate shuttle may be regarded as part of this metabolism and AOAA has been shown to produce striatal lesions characteristic of neurodegenerative diseases [33]. Furthermore, inhibition of the malate–aspartate shuttle has been reported in neurological disease [34–36].

4.5. *Biosynthesis of neurotransmitter glutamate is inhibited by phenylsuccinate and AOAA indicating involvement of the malate–aspartate shuttle*

Palaiologos et al. [23] demonstrated that inhibition of the malate–aspartate shuttle with AOAA or inhibition of the malate–α-ketoglutarate carrier by phenylsuccinate significantly decreased the biosynthesis of neurotransmitter glutamate by synaptosomes. These data confirm that a functioning malate–aspartate shuttle is necessary for synthesis of neurotransmitter glutamate. As noted above, phenylsuccinate was more effective than AOAA in inhibiting the Ca^{2+}-dependent depolarization coupled glutamate release from glutamatergic neurons utilizing exogenous glutamine as the precursor [23].

4.6. *BMA potently inhibits neuronal metabolism due, in part, to its effect on glutamate transport*

BMA was used to demonstrate a link between the activity of the malate–aspartate shuttle, oxygen consumption and the cytosolic NAD^+/NADH ratio in cerebral cortical slices [2]. BMA greatly inhibits glucose oxidation via pyruvate dehydrogenase and the TCA cycle, and aspartate aminotransferase activity, particularly in synaptic mitochondria [4]. BMA inhibited oxidation of glutamate, and decreased the intracellular concentration of aspartate and glutamine in cultured neurons [3]. However, some of the effects of inhibition with BMA may be due to the ability of this compound to potently inhibit glutamate uptake [19,21].

4.7. *3-NPA blocks oxaloacetate synthesis from glutamate (or other substrates) indirectly, so it blocks the malate–aspartate shuttle*

Since oxaloacetate cannot be generated by anaplerosis in neurons, continuous TCA cycle activity is required for maintenance of an appropriate concentration of oxaloacetate. The TCA cycle can be blocked at the level of succinate dehydrogenase using the competitive active site directed inhibitor 3-NPA as shown by Alston et al. [25]. This will lead to impairment

of malate–aspartate shuttle activity as shown by the decrease in the concentrations of glutamate and aspartate in the presence of 3-NPA in organotypic corticostriatal cultures [26].

It has been shown that 3-NPA caused a significant fall in ATP both in synaptosomes [37] and in neuronal cultures of murine frontal cortex [38]. Intrastriatal injection of 3-NPA has also been reported to result in a reduced ATP content within 3 h in the area close to the injection site [39]. 3-NPA inhibits synaptosomal respiration in a dose-dependent manner and led to increased lactate concentration [37]. With glutamine and 3-NPA in the medium, both glutamate and GABA increased inside the synaptosomes and the release of glutamate upon depolarization with veratradine and potassium rose as well [37]. In mice injected subcutaneously with 3-NPA an increased GABA concentration was observed, whereas glutamate was slightly decreased [40]. Furthermore, using [1-^{13}C]glucose or [2-^{13}C]acetate in combination with NMR spectroscopy, it could be shown that 3-NPA inhibited metabolism in neurons more than glial cells [41]. This is in agreement with the importance of the malate–aspartate shuttle in neurons.

To further explore this differential vulnerability the effect of blocking succinate dehydrogenase both in astrocytes and neurons was studied using [U-^{13}C]glutamate as a substrate [38,42]. In cerebellar granule neurons, TCA cycle activity was efficiently blocked by 3 mM 3-NPA [38] and metabolism of [U-^{13}C]glutamate was restricted to the formation of succinate. Aspartate labeling was abolished indicating an efficient block of the malate–aspartate shuttle. Only the uniformly labeled isotopomer of glutamate could be detected by NMR, indicating that there was no resynthesis of glutamate from TCA cycle intermediates [38]. The amount of labeled glutamate within the cells was decreased compared to control, which agrees well with the observations in synaptosomes [37,38]. Lactate labeling from TCA cycle intermediates as observed previously in vitro and in vivo [38,40], was abolished by 3 mM 3-NPA in cerebellar granule neurons [38].

4.8. *The malate–aspartate shuttle predominates in neurons and has a role in neurotransmitter biosynthesis*

Overall, evidence in the literature supports a key role of the malate–aspartate shuttle in synthesis of neurotransmitter glutamate and also in maintaining oxidative metabolism in neurons. Inhibition of this shuttle in neurons impairs metabolism and can result in neuronal death. Some studies provide evidence for the concept of an active glycerol 3-phosphate shuttle in neurons [5,13,27]. It is not surprising to find some role for the glycerol 3-phosphate shuttle in neurons in view of the importance of transferring reducing equivalents into the mitochondria in neurons in maintaining energy metabolism.

5. Astrocytes—effect of drugs

5.1. *Glycolysis is high in astrocytes so these cells need a way to reoxidize the NADH → NAD$^+$; therefore, some shuttle mechanism must be present*

The glycolytic pathway is active in astrocytes and can be upregulated under stress such as nitric oxide [43]. Although the malate–aspartate shuttle can be inhibited in astrocytes, oxidative metabolism is more difficult to inhibit than in neurons, strongly supporting the existence of an active glycerol 3-phosphate shuttle in these cells [13,15].

5.2. *The AGC1 carrier is present in astrocytes in vitro but may not be present in vivo*

A considerably lower level of AGC1 and malate–aspartate shuttle activity is present in astrocytes compared to the high amount in neuronal mitochondria [5]. It is, however, important to note that although this carrier is selectively enriched in neurons, low levels have been reported in astrocytes suggesting that a minimal malate–aspartate shuttle activity could be present [5]. Reports that AGC1 is upregulated in cultured astrocytes, and that citrin is found in glial cultures but not in brain, dictate that studies of malate–aspartate shuttle activity in these cells be interpreted with caution [5].

5.3. *Glycerol 3-phosphate dehydrogenase is believed to be localized in glia*

The expression and activity of glycerol 3-phosphate dehydrogenase is developmentally regulated in brain and increases dramatically during active myelination [44]. Although this enzyme is reported to be localized exclusively in oligodendroglial and Bergmann glial cells [44,45], glycerol 3-phosphate dehydrogenase activity has been reported in astrocytes [12,45], and may also be present in neurons [5]. It has been suggested that the pyruvate, which accumulates under severe hypoxia is channeled into alanine, and the increased NADH is reflected by the increase in glycerol 3-phosphate [46]. The report that glycerol 3-phosphate dehydrogenase increased >60-fold after focal cerebral ischemia is consistent with this concept [47]. This process is believed to take place in glia since glycerol 3-phosphate dehydrogenase has been shown to be present in oligodendrocytes and astrocytes [11,12]. It is conceivable that this pathway is also used in astrocytes during normoxia to some extent, when glycerol 3-phosphate levels will be kept low by the glycerol 3-phosphate shuttle and subsequent oxidative metabolism of NADH. That this may indeed be the case is indicated by the finding that glucose and lactate homeostasis which depends on the ability to regenerate NAD$^+$ in the cytosol was found to be only marginally affected by 3-NPA in astrocytes [15]. The most likely alternative shuttle mechanism is the one utilizing the cytosolic and mitochondrial forms of glycerol 3-phosphate dehydrogenase [15]. However, there are reports that the two isoforms of this enzyme have different cellular expression patterns, the cytosolic form being predominantly glial [11] and the mitochondrial form being neuronal [7]. It has, therefore, been claimed that this particular shuttle mechanism may be of little importance for the cytosolic-mitochondrial shuttling of reducing equivalents in brain [7]. However, it remains clear that this shuttle does take place in astrocytes in the presence of 3-NPA, which would inhibit malate–aspartate shuttle activity [15]. Moreover, oxidative metabolism of glucose and lactate in astrocytes takes place in the presence of AOAA, again suggesting that a shuttle mechanism other than the malate–aspartate shuttle which is inhibited by AOAA, is likely to be operating [13].

5.4. *Glucose and lactate oxidation in rat astrocytes are not dependent on the malate–aspartate shuttle*

In astrocytes incubated with 5 mM AOAA the oxidation of lactate was increased 36%, and the oxidation of glucose was increased ~3-fold over controls under these conditions that would inhibit malate–aspartate shuttle activity [13]. These findings demonstrate that oxidative metabolism in astrocytes must be linked to the glycerol 3-phosphate shuttle [13]. Different conclusions with regard to the significance and mechanism of glutamate metabolism through the TCA cycle have been reached. In one study, it could be demonstrated that the extent of oxidation is coupled to the glutamate concentration [48]. A prerequisite for entry of exogenous glutamate into the TCA cycle is the conversion of glutamate to α-ketoglutarate, which can take place via transamination or deamination. AOAA has been used to probe the significance of transamination for the oxidative metabolism of glutamate ([49,50] and references therein). As mentioned above, [U-^{13}C]glutamate can enter the TCA cycle and this may lead to the formation of ^{13}C labeled aspartate, lactate and in some cases also ^{13}C labeled citrate is observed [51]. Aspartate formation is dependent on transamination and, thus, will not take place in the presence of AOAA. Formation of lactate and citrate is, however, independent of transamination and only requires [U-^{13}C]glutamate entry into the TCA cycle. It could be shown that both lactate and citrate concentrations were unaffected by the presence of AOAA [50]. Thus, as originally proposed [20] it was shown that transamination played only a minor role for formation of α-ketoglutarate from glutamate [50]. However, this process appeared to be the major pathway for the opposite reaction, i.e. formation of glutamate from α-ketoglutarate. This is based on the finding that [1,2,3-^{13}C]glutamate and glutamine, formed from ^{13}C-labeled TCA cycle intermediates and unlabeled acetyl CoA, was strongly reduced in the presence of AOAA [50]. An explanation for the pronounced decrease of these isotope isomers (isotopomers) is that transamination is the major pathway for glutamate formation from α-ketoglutarate, which is in accordance with the low affinity of GDH for ammonia [52]. In contrast, incubation with AOAA inhibited glutamine oxidation in rat brain astrocytes by ~80%, demonstrating that entry of the glutamate formed endogenously from glutamine into the TCA cycle proceeded via transamination, not via glutamate dehydrogenase [13,49].

5.5. *3-NPA blocks oxaloacetate synthesis from glutamate (or other substrates) indirectly, so it blocks the malate–aspartate shuttle*

To probe if astrocytes are indeed less sensitive than neurons to 3-NPA due to less oxidative metabolism or possibly an additional pathway for transport of reducing equivalents, cultured astrocytes were incubated in the presence of two different concentrations of 3-NPA (3 and 10 mM) using [U-^{13}C]glutamate as the substrate [42]. For labeling patterns of [U-^{13}C]glutamate metabolism in astrocytes, see Bakken et al. [42]. As in neurons, 3-NPA clearly affected glutamate metabolism in astrocytes. Succinate accumulated intra- and extracellularly, and intracellular glutamate and glutamine concentrations were reduced. In the control group, the succinate concentration was too small to be detected by ^{13}C-NMR. After incubation with 3-NPA (both 3 and 10 mM) no label was detected in aspartate. However, label appeared in lactate in astrocytes receiving 3 mM 3-NPA, and intracellular [l,2,3-^{13}C]glutamate and extracellular [1,2,3-^{13}C]glutamine, resynthesized from TCA cycle intermediates, were also still present in cells receiving 3 mM 3-NPA, although both were significantly reduced. Such labeling from [U-^{13}C]glutamate is only possible using precursors from the TCA cycle, indicating that 3 mM 3-NPA was not sufficient to achieve a complete inhibition of the TCA cycle in astrocytes. With 10 mM 3-NPA, TCA cycle conversion of [U-^{13}C]glutamate to metabolites was restricted to the formation of succinate. Since, as noted above, in neurons a 3 mM concentration of 3-NPA was sufficient to block TCA cycle metabolism, it appears that astrocytes are more resistant to the effects of 3-NPA, in agreement with the study in mice [41].

Intrastriatal injection of 3-NPA has also been reported to result in a reduced ATP content within 3 h in the area close to the injection site [39]. In cultured cortical astrocytes a slight, but not significant, decrease in ATP was observed after incubation with 3-NPA [42]. These results suggest that astrocytic ATP stores are less vulnerable to 3-NPA intoxication than neuronal ATP stores. This may be due to use of the glycerol 3-phosphate shuttle for transporting reducing equivalents into mitochondria, and the glial localization of pyruvate carboxylase, which provides oxaloacetate and is essential for de novo synthesis of glutamine [15]. Indeed, there are indications of an increased pyruvate carboxylase activity in 3-NPA treated astrocytes since the glucose consumption was increased though lactate production was decreased [15,42]. In contrast, in synaptosomes, where the malate–aspartate shuttle appears to be important and pyruvate carboxylase is unlikely to be present, lactate concentration increased after 3-NPA treatment [37]. 3-NPA was additionally used to determine pyruvate carboxylation and metabolism of [3-^{13}C]malate in brain cells [15].

6. Gaps and future needs of research in this area

There have been relatively few studies of glycerol 3-phosphate shuttle activity in brain. However, several reports show evidence of this shuttle activity, or the ability of brain cells to support respiration with glycerol 3-phosphate as a substrate. However, using this substrate does not specifically address the question of whether cytosolic glycerol 3-phosphate dehydrogenase is present in neurons. Additional studies are needed using double labeling immunohistochemistry and confocal microscopy to obtain more information about the cellular localization of the isoforms of glycerol 3-phosphate dehydrogenase in brain tissue from different species. In depth studies on the cellular localization of aralar1 and citrin would clarify if either of these transporters are present on astrocyte mitochondria in vivo.

Pharmacological inhibitors of the malate–aspartate shuttle have been particularly useful for demonstrating the existence of this shuttle in brain, its role in neurotransmission and importance in neurons and synaptosomes. However, cur-

rently available inhibitors are limited by a lack of specificity for inhibiting aspartate aminotransferase and other mechanisms of action such as inhibiting glutamate uptake into brain cells. These limitations confound our ability to study the malate–aspartate shuttle and can lead to difficulty in interpreting experimental data. Studies with recently developed aralar-deficient mice demonstrate the importance of this carrier for the synthesis of neuronal aspartate and *N*-acetylaspartate (NAA), and for myelination in brain [53]. The development of these mice provides an important new tool for studying the role(s) of the malate–aspartate shuttle.

The lack of pharmacological agents to completely inhibit the glycerol 3-phosphate shuttle leads to difficulties in studying this shuttle in brain. However, many studies using inhibitors of the malate–aspartate shuttle provide evidence of glycerol 3-phosphate shuttle activity in brain cells. The development of highly specific inhibitors of both the malate–aspartate shuttle and the glycerol 3-phosphate shuttle would provide investigators with the pharmacological tools needed to advance the understanding of the roles, activity and relative importance of these shuttles in neurons and glial cells.

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