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Evidence Supporting the Existence of an Activity-Dependent Astrocyte-Neuron Lactate Shuttle

Abstract

Mounting evidence from in vitro experiments indicates that lactate is an efficient energy substrate for neurons and that it may significantly contribute to maintain synaptic transmission, particularly during periods of intense activity. Since lactate does not cross the blood-brain barrier easily, blood-borne lactate cannot be a significant source. In vitro studies by several laboratories indicate that astrocytes release large amounts of lactate. In 1994, we proposed a mechanism whereby lactate could be produced by astrocytes in an activity-dependent, glutamate-mediated manner. Over the last 2 years we have obtained further evidence supporting the notion that a transfer of lactate from astrocytes to neurons might indeed take place. In this article, we first review data showing the presence of mRNA encoding for two monocarboxylate transporters, MCT1 and MCT2, in the adult mouse brain. Second, by using monoclonal antibodies selectively directed against the two distinct lactate dehydrogenase isoforms, LDH₁ and LDH₅, a specific cellular distribution between neurons and astrocytes is revealed which suggests that a population of astrocytes is a lactate ‘source’ while neurons may be a lactate ‘sink’. Third, we provide biochemical evidence that lactate is interchangeable with glucose to support oxidative metabolism in cortical neurons. This set of data is consistent with the existence of an activity-dependent astrocyte-neuron lactate shuttle for the supply of energy substrates to neurons.

Key Words

Glia
Monocarboxylates
Lactate dehydrogenase
Glutamate
Glucose
Transporters
 Na^+/K^+ -ATPase

Introduction

A fundamental principle in the physiology of brain energy metabolism is that neuronal activity is tightly coupled to blood flow and energy metabolism. Since in normal conditions, glucose is the exclusive energy substrate for the brain, from the metabolic point of view, this coupling implies that spatially localized and temporally re-

stricted increases over basal glucose utilization occur in register with neuronal activity in brain areas subserving specific modalities. In order to elucidate the physiological bases of this coupling it is crucial to better understand the cellular and molecular bases of brain energy metabolism and in particular the nature of the signals produced by neuronal activity which lead to the localized increases in glucose utilization.

Over the last few years it has become clear that highly regulated metabolic interactions occur between neurons and astrocytes, providing a conceptual framework for the interpretation of new data on the molecular aspects of brain energy metabolism [for review, see Tsacopoulos and Magistretti, 1996]. On the basis of in vitro observations obtained in primary astrocyte cultures, our laboratory proposed in 1994 an operational model that could account for the coupling of neuronal activity to energy metabolism [Pellerin and Magistretti, 1994]. Central to this model is the role of glutamate released during synaptic activity and its reuptake via specific transporters into astrocytic processes that ensheathe synaptic contacts. We have indeed shown that the sodium-dependent transport of glutamate into astrocytes triggers aerobic glycolysis, i.e. glucose uptake and lactate production, in this cell type. A key role in coupling glutamate uptake to glucose metabolism in astrocytes is played by the Na^+/K^+ -ATPase, and in particular by its α_2 subunit, mobilized by the increased Na^+ influx which is coupled to glutamate transport [Pellerin and Magistretti, 1996, 1997]. In addition to ensheathing synapses, astrocytes possess specialized processes, the end-feet, which cover virtually all intraparenchymal capillaries. These specialized processes are enriched in glucose transporter Glut 1 [Morgello et al., 1995]. This cytological arrangement taken together with the functional observations briefly reviewed, strongly suggests that neuronal activity resulting in synaptic release of glutamate triggers the entry of glucose into the brain parenchyma at the level of astrocytes, thus providing a simple and direct mechanism coupling neuronal activity to energy metabolism. Recent evidence obtained *in vivo* by NMR spectroscopy supports this model [see Sibson et al., 1998 and this issue].

While this model could provide a coherent explanation at the cellular and molecular levels for the activity-dependent local import of glucose into the brain parenchyma, the question of the metabolic fate of glucose and of the substrate(s) released by astrocytes to support neuronal activity needs further consideration. Over the last 2 years, we have addressed this specific question and obtained supporting evidence for the existence of an activity-dependent lactate shuttle between astrocytes and neurons. In this article we review the evidence and present some recent functional observations which further support the notion that lactate is indeed an efficient substrate for neurons.

Material and Methods

Cloning of cDNA Fragments for Mouse Monocarboxylate Transporters MCT1 and MCT2

Cloning has been performed as described in Bröer et al. [1997]. Briefly, the MCT1 and MCT2 cDNA fragments were obtained by reverse transcription and PCR amplification of poly (A)⁺ mRNA isolated from primary cultures of mouse cortical neurons and mouse liver respectively. The RT-PCR fragments were subcloned in pT7Blue(R) vector (Novagen). The identity of the amplified MCT1 and MCT2 cDNA fragments was confirmed by sequencing using an automated DNA sequencer (ALF DNA analysis system, Pharmacia). The amplified MCT1 cDNA fragment shares 89% nucleotide identity with the hamster MCT1 cDNA sequence [Kim et al., 1992; Kim Garcia et al., 1994] and was later found to be identical to the mouse MCT1 cDNA sequence [Carpenter et al., 1996]. The amplified MCT2 cDNA fragment was shown to share 84% nucleotide identity with the hamster MCT2 cDNA sequence [Kim Garcia et al., 1995].

Northern Blot Analysis of MCT1 and MCT2

The detailed procedure for Northern blotting of MCTs mRNA has been described in Pellerin et al. [1998]. Total RNA was extracted using the CsCl centrifugation procedure as described by Chirgwin et al. [1979]. Poly (A)⁺ RNA was obtained by passing total RNA through an oligo(dT)-cellulose spin column (Pharmacia). 20 μg of total RNA or 4 μg of poly (A)⁺ RNA were electrophoresed on a denaturing 1.2% agarose gel containing 2 M formaldehyde, and were transferred onto nylon membrane (Gene Screen, DuPont-NEN, Boston, Mass., USA). Hybridization was performed for 16–18 h at 65°C in 50% formamide, 5 \times SSC, 1 \times PE, 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 0.2% bovine serum albumin, and 150 $\mu\text{g}/\text{ml}$ sheared denatured salmon sperm DNA, which contained a ^{32}P MCT1 or MCT2 riboprobe. Filters were then washed under high-stringency conditions (twice with 2 \times SSC/0.1% SDS at 65°C for 15 min and once with 0.1 \times SSC/0.1% SDS at 65°C for 15 min), and apposed to Kodak AR film at –70°C with an intensifying screen.

In situ Hybridization for MCT1 and MCT2

The detailed procedure for *in situ* hybridization was described in Pellerin et al. [1998]. Plasmids containing MCT1 or MCT2 cDNA fragments inserted in the pT7Blue(R) vector in both orientations were linearized with either *Bam*H (MCT1) or *Eco*I (MCT2). Digoxigenin (DIG)-labeled antisense- or sense-strand RNA probes were prepared by *in vitro* transcription with T7-RNA polymerase (Boehringer Mannheim). Sections were incubated overnight at 58°C in the prehybridization buffer containing 200 ng/ml of sense or antisense DIG-labeled RNA probe. At the end of the incubation period, sections were washed successively in 2 \times SSC for 30 min at room temperature followed by 1 h at 65°C and finally in 0.1 \times SSC for 1 h at 65°C. Immunodetection of the hybridized probe was performed using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Production of Monoclonal Antibodies and Immunohistochemistry for LDH

The procedure was as described previously [Bittar et al., 1996] except for the last steps to obtain the monoclonal antibodies. Lactate dehydrogenase isoenzyme 1 and 5 from rabbit heart and muscle respectively were purchased from Sigma (Buchs, Switzerland). Both

products were dialyzed overnight against H₂O at 4°C and subsequently stored lyophilized. Wistar male rats were immunized as follows: on days 0 and 15, subcutaneous injections of immunogens were given with the same volume of complete Freund's adjuvant (FA), and three booster injections were given intraperitoneally with incomplete FA on days 30, 45 and 60. Blood was removed from the tails by puncture 10 days after the last injection, serum was immediately extracted by centrifugation and stored at 4°C in the presence of minute amounts of sodium azide. A first screening for the presence of antibodies was performed by immunohistochemistry in human occipital cortex for both antisera. Responding animals were boosted intraperitoneally with 0.2 mg immunogen in 0.3 ml saline without FA. Three days later, hybridomas were produced by fusion of splenocytes with log-phase Sp2 myeloma cells in the presence of polyethylene glycol. The fused cells were cloned by limiting dilution in RPMI 1640 containing 15% fetal calf serum and HAT medium (Gibco Brl, Basel, Switzerland). Ten to 15 days later, supernatant screening was performed by immunohistochemistry in human occipital sections.

Supernatants containing 3% bovine serum albumin (BSA) and 5% Triton X-100 were deposited on human brain cryostat sections 20 µm thick overnight at 4°C. Following incubation, sections were rinsed in PBS and allowed to incubate for 1 h with rabbit anti-rat IgG conjugated to peroxidase (Dako, Zug, Switzerland) at a working dilution of 1:200. Immunoreaction products were visualized by exposing the sections to a solution of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.005% H₂O₂ in PBS for 20 min at room temperature.

Neuronal Cultures and Measurement of Mitochondrial Activity

Neuronal cultures from cerebral cortex 17-day-old mouse embryos were prepared as previously described [Stella et al., 1995]. After 5–7 days in vitro cortical neurons were rinsed twice with a media without any energy substrate (composition in mM: NaCl, 145; KCl, 5.5; CaCl₂, 1.1; MgCl₂, 1.1; NaHCO₃, 3.6; HEPES, 20; pH, 7.4; 1 ml/well). Cells were then incubated for 3 h at 37°C in the same buffer containing either glucose or lactate (Sigma) at the desired concentration. At the end of the 3-hour incubation period, 100 µl of the same buffer containing 5 mg MTT/ml was added in each well for an additional 3 h. Supernatant was then removed and the precipitated formazan was dissolved with 1 ml dimethyl sulfoxide followed by gentle shaking of the dishes. Optical density of dissolved formazan was measured at 560 nm. MTT and αCNN were obtained from Sigma.

Lactate Transporters

As indicated in the Introduction, evidence has been provided for a mechanism whereby glucose taken up by glial cells as a result of an activity-dependent signal (e.g. glutamate) is converted to lactate [Pellerin and Magistretti, 1994; Poiry-Yamate et al., 1995]. This has raised the possibility that lactate is the activity-dependent metabolic substrate provided by astrocytes to neurons [Pellerin and Magistretti, 1994; Poiry-Yamate et al., 1995]. Indeed, several studies have reported the ability of lactate to sustain synaptic activity, to protect neurons from glucose deprivation or to allow neurons to recover from hypoxic

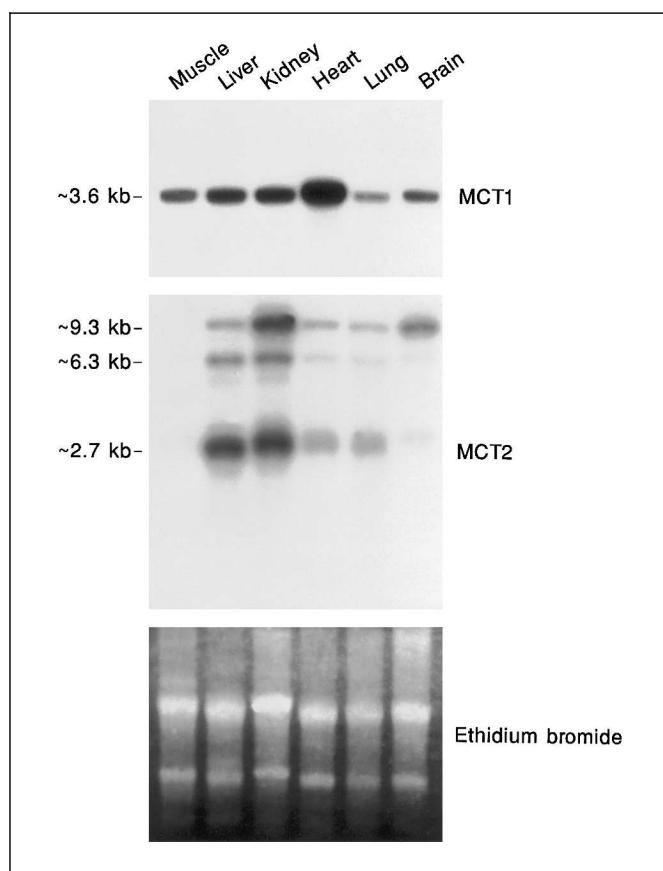


Fig. 1. Northern blot analysis of MCT1 and MCT2 expression in various mouse tissues including brain. 20 µg of total RNA were extracted from each tissue, electrophoresed and hybridized with an antisense ³²P-MCT1 or MCT2 riboprobe. Exposure time was 1 day for MCT1 and 3 days for MCT2.

episodes [McIlwain, 1953; Schurr et al., 1988, 1997; Iizumi et al., 1997]. In order to provide an intercellularly exchangeable energy substrate, lactate must be transported across membranes since it does not readily diffuse through them. Recently, two transporters, MCT1 and MCT2 for monocarboxylates (which include lactate, pyruvate and the ketone bodies β-hydroxybutyrate and acetocetate) have been identified and their distribution in peripheral tissues in the hamster has been described [Kim et al., 1992; Kim Garcia et al., 1994, 1995]. More recently a third monocarboxylate transporter has been described, MCT3, which appears to be expressed only in the retinal epithelium [Philp et al., 1995; Yoon et al., 1997].

Using specific probes we have examined by Northern blot the mRNA distribution for MCT1 and MCT2 in various tissues of the adult mouse, including the brain. As shown in figure 1, MCT1 mRNA was present as a single



Fig. 2. Localization by *in situ* hybridization of the MCT1 mRNA in the adult mouse brain. Sagittal sections were hybridized with either an antisense MCT1 (**A**) or sense MCT1 (**B**) digoxigenin-labeled RNA probe. Note that only the antisense probe should bind and give a signal, since it has a complementary sequence to the target mRNA. The sense probe, which has the same sequence as the target mRNA, will not bind and is used as a negative control.

transcript of approximately 3.6 kb and was expressed in all tissues examined. It was particularly abundant in the heart and displayed a low level of expression in the lungs. The level of expression in the brain was comparable to that observed in muscle. MCT2 expression was much less abundant than MCT1, as indicated by the fact that it required a longer exposure time in order to be revealed. Three transcripts were detected with lengths of approximately 9.3, 6.3 and 2.7 kb, respectively. Expression of MCT2 was abundant in liver and kidney and undetectable in muscle. In the brain, the 9.3-kb transcript was predominant.

To obtain more information about the regional expression of monocarboxylate transporters in the central ner-

vous system, we performed an *in situ* hybridization study. As shown in figure 2A, MCT1 mRNA was strongly expressed throughout the cortex (except in layer I), in the hippocampus and in the cerebellum. In the hippocampus, the pyramidal and granule cell layers appeared heavily labeled while in the cerebellum the granular layer was densely stained. At a higher magnification, strong expression was found to be associated with the Purkinje cell layer (not shown). Subcortical structures, such as the striatum, were also labeled but to a lesser extent. Very little staining was obtained with the sense probe (fig. 2B).

The expression of MCT2 mRNA appeared less intense than that of MCT1 (fig. 3A). Both the pyramidal and



Fig. 3. Localization by *in situ* hybridization of the MCT2 mRNA in the adult mouse brain. Sagittal sections were hybridized with either an antisense MCT2 (**A**) or sense MCT2 (**B**) digoxigenin-labeled RNA probe. Note that only the antisense probe should bind and give a signal, since it has a complementary sequence to the target mRNA. The sense probe, which has the same sequence as the target mRNA, will not bind and is used as a negative control.

granule cell layers in the hippocampus were labeled as well as the granular layer of the cerebellum. Staining was also present throughout the cortex and in subcortical structures such as the striatum. As for MCT1, nonspecific staining with the sense probe was very low (fig. 3B).

Recent work using specific antibodies against MCT1 and MCT2 has confirmed the presence of these transporters in the brain [Gerhart et al., 1997, 1998]. These results on the distribution of MCTs provide a structural support to functional studies indicating the presence of lactate uptake systems on brain cells [Nedergaard and Goldman, 1993; Tildon et al., 1993; Bröer et al., 1997]. In addition, evidence for the expression of monocarboxylate trans-

porter mRNAs within the brain parenchyma in adult animals, despite their seemingly complete absence on capillaries [Pellerin et al., 1998], is consistent with the concept that lactate may represent an important energy substrate transferred from astrocytes to neurons [McKenna et al., 1993; Bittar et al., 1996].

Lactate Dehydrogenase Isoenzymes

Biochemically, lactate is a metabolic dead end. In order to be used as an energy substrate, it must be converted to pyruvate, which then constitutes an efficient

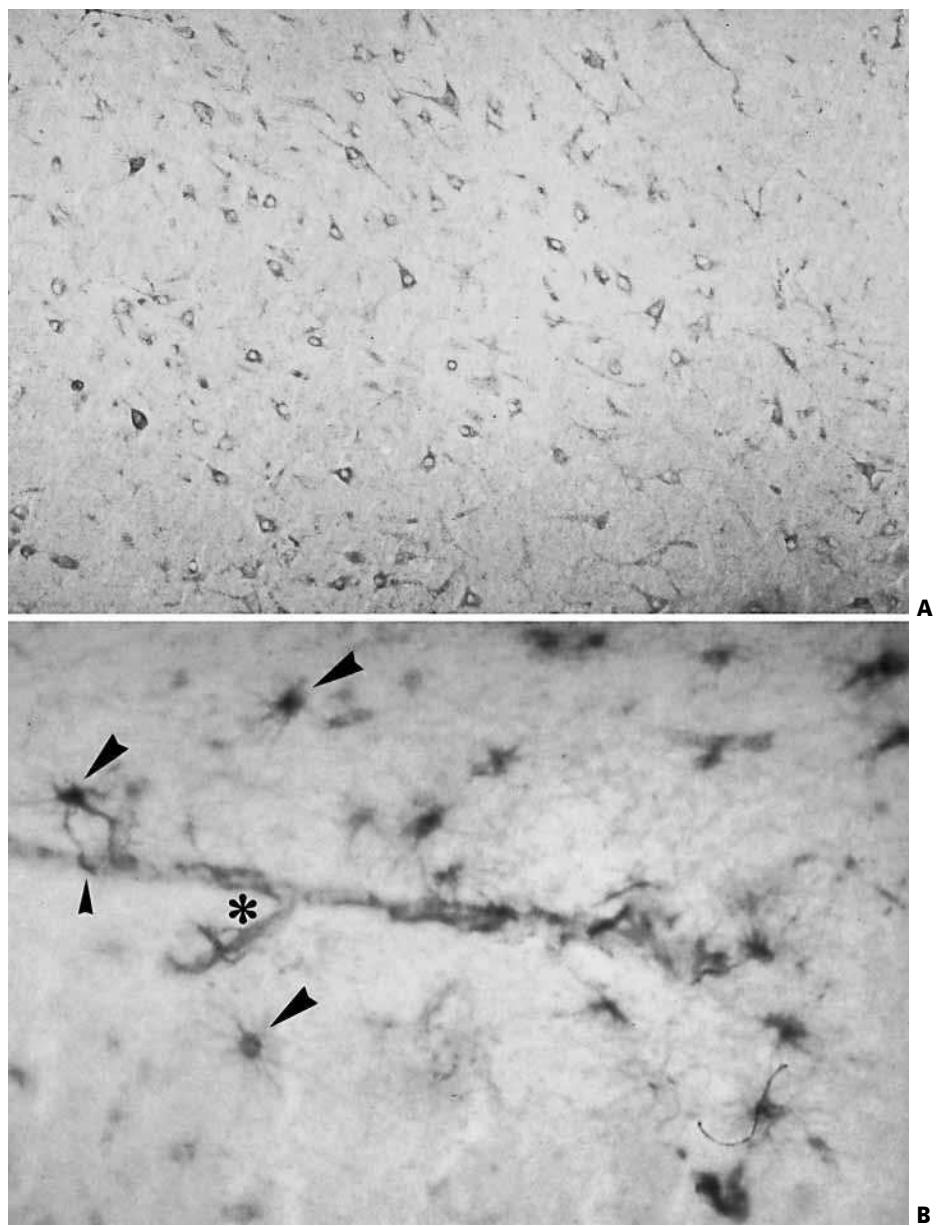


Fig. 4. Immunohistochemical localization of LDH isoforms in human brain. **(A)** Neurons in the CA4 area of the human hippocampus immunostained with an anti-LDH₁ monoclonal antibody. **(B)** Immunostaining of astrocytes in the human hippocampus with an anti-LDH₅ monoclonal antibody. Notice the astrocyte on the left-hand side with labeled end-feet contacting the blood vessel. Large arrows = Immunostained astrocytes, small arrow = astrocytic end-foot; asterisk = blood-vessel.

metabolic fuel by providing 18 ATPs per molecule. The enzyme responsible for the interconversion of lactate to pyruvate is lactate dehydrogenase (LDH). It is expressed in various tissues as five tetrameric forms. The muscle form (M or LDH₅) is composed of four identical muscle type subunits while the heart form (H or LDH₁) of four heart type subunits. The other three isoforms (LDH₂, LDH₃, LDH₄) are heterogenous trimers made of both muscle and heart type subunits. We have raised monoclonal antibodies specific for LDH₁ and LDH₅ respective-

ly, to characterize by immunohistochemistry the cellular distribution of these isoforms in the human brain. The immunostaining pattern revealed that neurons were heavily labeled by the anti-LDH₁ antibody as illustrated in figure 4A. Some astrocytes were also stained by this antibody (not shown). In contrast, using an antibody against the LDH₅ form, neurons were never stained, while a population of astrocytes was strongly labeled (fig. 4B). Both the cell bodies and processes of astrocytes were stained. The resolution obtained with the antibody

recognizing LDH₅ provided a rather detailed illustration of the tight relationship existing between astrocytic end-feet and capillaries, as illustrated in figure 4B.

It is well established that tissues which display intense but brief increases in activity (e.g. fast-twitch fibers in muscles) sustain high glycolytic rates and produce large amounts of lactate. Such tissues tend to contain higher proportions of the LDH₅ isoform. The reason is that this isoform has a greater maximal velocity (V_{max}) than LDH₁ [Gerhardt-Hansen, 1968], thus providing a better capacity to convert pyruvate to lactate, an expected feature to support high glycolytic rates. In contrast, the LDH₁ isoform is preferentially expressed in tissues such as the heart, which are characterized by a more sustained level of activity. The LDH₁ isoform exhibits a lower K_m and is inhibited by pyruvate and lactate at relatively low concentrations [Gerhardt-Hansen, 1968; Bishop et al., 1972]. These characteristics render LDH₁ poised to promote the conversion, with high affinity, of a substrate (lactate) into a product (pyruvate) which requires efficient and rapid clearance. These conditions are met in oxidative tissues, such as the heart which maintains its pyruvate concentration low and consumes lactate to face energy demands. In other words, cells expressing high levels of LDH₅ are likely to display high glycolytic rates and to produce large amounts of lactate while those in which LDH₁ is preferentially expressed are endowed with the capacity to utilize lactate as an energy substrate. Immunocytochemical data reported here and in Bittar et al. [1996] suggest that astrocytes contain the LDH isoform which is efficient to favor lactate production, while neurons express an isoform which favors lactate utilization. These features would define neurons as lactate ‘sinks’ within the brain parenchyma as opposed to astrocytes which would act as lactate ‘sources’.

Lactate as Substrate for Oxidative Metabolism in Neurons

As mentioned earlier, there is ample electrophysiological evidence that lactate is an efficient energy substrate for neurons [Schurr et al., 1988, 1997; Izumi et al., 1997]. To complement these observations, we have explored further with a biochemical approach the role of lactate as a substrate for oxidative metabolism in neurons. For this purpose, we have used a simple colorimetric assay which provides an index of the TCA cycle activity. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is transformed following activation of mitochondrial dehy-

Table 1. Support of oxidative metabolism in cortical neurons by glucose or lactate

Conditions	MTT activity ^a , %
Glucose 1 mM	100.0±2.0
Lactate 2 mM	114±0.6
Glucose 10 mM	209.0±2.2
Lactate 10 mM	195.0±0.2
Lactate 2 mM + αCNC 0.3 mM	47.0±1.0
Lactate 2 mM + αCNC 3 mM	12.0±1.4

αCNC = αCyano-4-hydroxycinnamate.

^a MTT activity reported as percentage of activity measured in presence of 1 mM glucose.

drogenases [Slater et al., 1963]. It is converted to formazan which forms an insoluble blue precipitate. Accumulation of this precipitate, which can be quantitated spectrophotometrically, is directly proportional to the activity of mitochondrial dehydrogenases, and by extension, to TCA cycle turnover and oxidative metabolism. Using this method as an index of oxidative metabolism, we have observed that lactate (2 mM) and glucose (1 mM) produce an equivalent MTT signal in neurons (table 1). Maximal level of MTT activity, which was reached at 10 mM for both glucose and lactate, was also comparable between the two substrates. In order to assess the involvement of monocarboxylate transporters for the utilization of lactate as an energy substrate by neurons, we have used α-cyano-4-hydroxycinnamate (αCNC), a selective inhibitor of lactate transporters. As shown in table 1, αCNC at the concentration of 3 mM decreases MTT activity supported by lactate by more than 85%. In summary, these observations indicate that lactate alone fully substitutes for glucose as a metabolic substrate for oxidative metabolism in cortical neurons and that monocarboxylate transporters are essential to ensure efficient utilization of lactate. These data are consistent with previous studies indicating that in synaptosomes lactate sustains rates of oxidation which can be superior to those observed with glucose [McKenna et al., 1993, 1994]. They are also consistent with the observations by Schurr et al. [1988, 1997] that lactate alone can fully support synaptic transmission. Finally, these data do not exclude the possibility that a fraction of lactate imported into neurons could also provide a carbon source for the synthesis of amino acid neurotransmitters such as glutamate or GABA, as recent NMR data suggest [Waagepetersen et al., 1998].

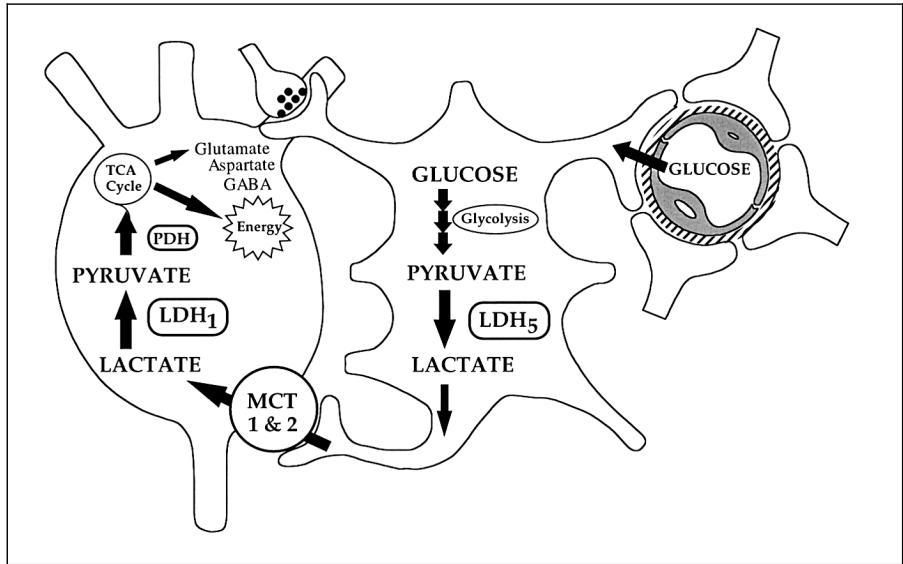


Fig. 5. Schematic representation of the proposed astrocyte-neuron lactate shuttle. Following neuronal activation and synaptic glutamate release, glutamate reuptake into astrocytes triggers increased glucose uptake from capillaries via activation of an isoform of the Na^+/K^+ -ATPase which is highly sensitive to ouabain, possibly the α_2 isoform [Pellerin and Magistretti, 1994, 1997]. Glucose is then processed glycolytically to lactate by astrocytes which are enriched in the muscle form of LDH (LDH₅). The exchange of lactate between astro-

cytes and neurons is operated by monocarboxylate transporters. Lactate is then converted to pyruvate since neurons contain the heart form of LDH (LDH₁). Pyruvate, via the formation of acetyl CoA by pyruvate dehydrogenase (PDH), enters the TCA cycle thus generating 18 ATP/lactate. It is also conceivable that some of the carbons in the lactate molecule could enter into amino acid pools such as the neurotransmitters glutamate, aspartate and GABA as recently demonstrated by NMR [Waagepetersen et al., 1998].

Overview

An array of in vitro experimental data accumulated over the years has indicated that lactate is an efficient energy substrate to support neuronal activity and survival [McIlwain, 1953; Schurr et al., 1988, 1997; Izumi et al., 1997], and in some circumstances it might even be preferred to glucose [Larrabee, 1995]. In vivo, blood-borne lactate cannot represent an efficient energy substrate for the adult brain, since it only marginally crosses the blood-brain barrier [Pardridge, 1977]. However, if formed *within* the brain parenchyma, lactate may indeed meet the energy requirements of neurons, particularly during periods of intense activity.

We have previously described a mechanism accounting for the coupling between neuronal activity and glucose utilization [Pellerin and Magistretti, 1994]. Reuptake of synaptically-released glutamate into astrocytes activates aerobic glycolysis in this cell type via a stimulation of a specific isoform of the Na^+/K^+ -ATPase which is highly sensitive to ouabain [Pellerin and Magistretti, 1996, 1997]. Lactate, which is released in large amounts by astrocytes [Walz and Mukerji, 1988], was the major glyco-

lytic intermediate produced in this situation, thus suggesting that it might constitute the energetic substrate transferred from astrocytes to neurons. Three conditions have to be fulfilled in order to support this possibility. First, since lactate does not cross easily cell membranes, a specific transport system must be demonstrated in the central nervous system. Second, lactate needs to be converted to pyruvate to become a useful energy substrate. This reaction is catalyzed by LDH which exists as different isoforms distributed according to the glycolytic or oxidative nature of the cellular system. Finally, biochemical evidence should be obtained to confirm that lactate can support oxidative metabolism in neurons. Data reviewed here provide evidence for all three conditions thus supporting the existence of an astrocyte-neuron lactate shuttle (fig. 5). According to this model, following neuronal activation and glutamate-induced astrocytic glucose uptake from the capillaries, glucose is processed glycolytically and lactate released. Intercellular exchange of lactate is operated by specific monocarboxylate transporters; within neurons lactate is transformed into pyruvate by LDH₁ to sustain oxidative metabolism. Furthermore, recent data obtained *in vivo* with implanted lactate and glucose

microsensors together with an oxygen electrode in the hippocampus of the rat strongly support the proposed concept that lactate, formed from glucose by astrocytes in an activity-dependent manner, constitutes the preferred energy substrate for oxidation during periods of neuronal activation [Hu and Wilson, 1997].

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