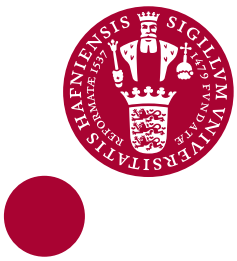

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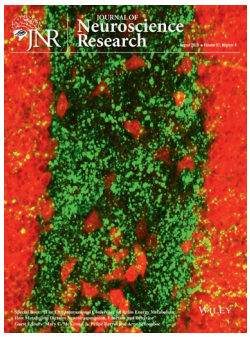

Astrocytic pyruvate carboxylation: Status after 35 years

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Abstract

The first two publications dealing with the question of the cellular localization of the enzyme pyruvate carboxylase (PC) which in the brain represents the most important metabolic pathway to allow anaplerosis of TCA cycle constituents were published in 1983 and 1985. Hence, 2018 marks the 35th anniversary of the notion based on the results of the publications provided above that PC-catalyzed anaplerosis in the brain is an astrocytic process. This review will provide the background for investigating this enzymatic pathway as well as a discussion of cataplerosis, the degradation of products from anaplerosis, and the current status of the functional significance of pyruvate carboxylation in brain metabolism.

KEYWORDS
anaplerosis, astrocytes, cataplerosis, glutamate, glutamine, neurons

1 | INTRODUCTION

Glucose, the main energy substrate of the brain (see McKenna, Dienel, Sonnewald, Waagepetersen, & Schousboe, 2012), is converted to pyruvate in the glycolytic pathway, which can either be reduced to lactate by lactate dehydrogenase or oxidized to acetyl-CoA by pyruvate dehydrogenase. In the latter case, further oxidation to CO_2 takes place in the tricarboxylic acid (TCA) cycle. The reactions in the TCA cycle represent the key processes in the generation of chemical energy in the form of ATP since the reduced co-enzymes generated by the oxidative processes of the cycle are utilized by the respiratory chain to convert ADP to ATP (for references, see McKenna et al., 2012). In addition to its role in energy production, the TCA cycle can be considered a biosynthetic pathway since some of its intermediary constituents such as oxaloacetate and α-ketoglutarate can be converted primarily to aspartate and glutamate from which glutamine and GABA can be produced. Removal of these TCA cycle keto-acids represents a drain of the TCA cycle constituents and hence, they must be replaced thereby allowing the continued function of this essential pathway. The process needed for this replacement is termed anaplerosis and this process requiring the enzyme pyruvate carboxylase (PC) constitutes the topic of this review. Labeled glucose has been used extensively to analyze the synthesis of these compounds representing the anaplerotic process.

2 | DISCUSSION

2.1 | Early studies of glutamate metabolism and synthesis using radiolabeled glucose

Studies by Berl, Nicklas, and Clarke (1968) and Berl and Clarke (1969) using radiolabeled glucose and other substrates for brain metabolism had pointed out that glutamate metabolism must be complex possibly reflecting the cellular composition of the brain by neurons and astrocytes likely exchanging metabolites. Thus, when [^{14}C]glucose was the substrate, the radioactive labeling of glutamate and glutamine followed the classical precursor–product relationship in which the product (glutamine) has a lower specific radioactivity than that of its precursor (glutamate). On the contrary, when [^{14}C]acetate was used as the substrate, the opposite relationship was found, i.e., the specific radioactivity of glutamine was higher than that of glutamate. This can only be explained assuming at least two metabolic pools of glutamate having different turnover rates concerning the conversion to glutamine as

Significance

The demonstration of a preferential if not exclusive non-neuronal, glial expression of the enzyme pyruvate carboxylase has facilitated the conclusion that only glial cells are capable of performing a net synthesis of the precursor for neuronal synthesis of the neurotransmitter amino acids: glutamate and GABA. Hence, a glial–neuronal exchange of metabolites is mandatory for the maintenance of the neurotransmission processes mediated by these two amino acids, processes that govern an overwhelming fraction of neurotransmission. The review provides the background for understanding the importance of this process. An example of the importance of this anaplerotic process is its involvement in the amelioration of the effects of hepatic encephalopathy, allowing fixation of excess ammonia as the amide group in glutamine synthesized in astrocytes.

pointed out by Garfinkel (1966), Berl et al. (1968) and van den Berg and Garfinkel (1971). This led to the notion that the cellular composition of the brain, i.e., neurons and glial cells, would allow an exchange between the cellular compartments of the two amino acids in question (Balazs, Machiyama, & Patel, 1973). However, it was not until the discovery that the glutamine synthesizing enzyme, glutamine synthetase (GS), is astrocyte specific (Martinez-Hernandez, Bell, & Norenberg, 1977) that the glutamate–glutamine cycle involving astrocytes and glutamatergic neurons could be proposed (see Cotman, Foster, & Lanthorn, 1981).

2.2 | Misconceptions regarding net synthesis based on labeling

Another important finding regarding the radiolabeling of glutamate from [^{14}C]glucose was that the labeling in glutamate was extremely rapid (Berl et al., 1968). Since the use of radiolabeling with ^{14}C does not easily allow the determination of the specific carbons being labeled in the metabolites generated from, e.g., [^{14}C]glucose, a rapid labeling may be interpreted as a net synthesis. Since glutamate was considered a neurotransmitter (Curtis, Phillis, & Watkins, 1959), the general assumption based on its rapid labeling from[^{14}C]glucose was that neurons could perform a net synthesis of glutamate from glucose (see Shank & Campbell, 1983). However, it is important to distinguish between, on the one hand, cycling of label from metabolites, which does not change the concentration and, on the other hand, net synthesis, which may increase or maintain the amount of metabolites, possibly as a result of depletion of a particular metabolite. In the former case, carbon skeletons are preserved to a certain extent, and in the latter they are lost from the system, which requires a de novo synthesis to maintain the original content.

2.3 | Need for CO_2 fixation to allow net synthesis of glutamate

The carbon skeleton of glutamate originates metabolically from α-ketoglutarate, a key intermediary of the TCA cycle and hence, a net synthesis of glutamate will lead to a depletion of TCA cycle constituents (see McKenna et al., 2016). In order for the cycle to maintain its operation, there must be a mechanism by which a de novo synthesis of TCA cycle constituents can be performed, i.e., an anaplerotic metabolic pathway. Three enzymes are capable of catalyzing such a pathway, namely malic enzyme (ME), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate carboxylase (PC). In the brain, the reaction catalyzed by PC is the quantitatively most prominent (Patel, 1974). The availability of the substrate carbon dioxide/bicarbonate as well as the obligatory activator, acetyl-CoA, represents the main regulatory entities for the enzyme. It was therefore of interest to establish the cellular localization of this enzyme and almost at the same time two research groups set out to investigate this, using different approaches (Shank, Bennett, Freytag, & Campbell, 1985; Yu, Drejer, Hertz, & Schousboe, 1983). Figure 1 shows a schematic presentation of the processes involved in anaplerosis.

2.4 | Studies aimed at determining the cellular localization of CO_2 fixation, the role of pyruvate carboxylation, and the role of neurons versus astrocytes

The study by Yu et al. (1983) utilized the availability of highly purified and functionally intact primary cultures of astrocytes and glutamatergic as well as GABAergic neurons to measure the enzymatic activity of PC in the cells and as a control in homogenates from brains of new-born and adult animals. The PC activity was found to be higher in the cultured astrocytes than in homogenates from adult brain and at the same time the PC activity was below the detection limit in the cultured neurons. On the basis of this, it was concluded that PC must be an astrocyte-specific enzyme not expressed in neurons. Shortly after, Shank et al. (1985) using a PC-specific antibody and immunocytochemical staining of brain slices also showed astrocyte-specific expression of the enzyme, a finding confirmed 10 years later by Cesar and Hamprecht (1995) using monoclonal antibodies against rat and mouse PC. Another report summarizing the use of ^{13}C-labeled precursors also concluded that the pyruvate carboxylation process mainly if not exclusively takes place in astrocytes

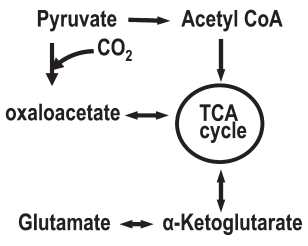

FIGURE 1 Schematic presentation of pyruvate carboxylation. Abbreviation: TCA, tricarboxylic acid

(Sonnewald & Rae, 2010). Altogether, this provides solid evidence that anaplerosis of the TCA cycle can only take place in the astrocytes and not in neurons. It should, however, be noted that pyruvate carboxylation was later found to take place in oligodendrocytes (Amaral, Hadera, Tavares, Kotter, & Sonnewald, 2016).

2.5 | Discussions of the role of the glutamate/glutamine cycle regarding its quantitative stoichiometry

The above findings concern the cellular localization of the two enzymes, PC and GS, the combined action of which allows a net synthesis of glutamine to be performed, being a part of the glutamate–glutamine cycling between neurons and astrocytes. This additionally explains the compartmentation of the glutamate pool indicated by the metabolic studies mentioned above. The cycle was originally conceived as a stoichiometric (1:1) exchange of glutamate release from neurons as a neurotransmitter from glutamatergic neurons with glutamine produced in astrocytes after the uptake of the transmitter glutamate (Cotman et al., 1981). In this context, it should be mentioned that it had been shown that astrocytes expressed a highly efficient high affinity uptake system for glutamate (Hertz, Schousboe, Boechler, Mukerji, & Fedoroff, 1978; Schousboe, Svenneby, & Hertz, 1977), a notion confirmed by the advent of cloning of the transporter and production of specific antibodies raised against the major types of these transporters (Danbolt, 2001).

If indeed the glutamate–glutamine cycle were to operate in a strict 1:1 stoichiometric fashion, glutamate taken up into astrocytes from the synaptic cleft would need to be quantitatively converted to glutamine in the GS-catalyzed reaction, amidating glutamate at the cost of ATP. Several studies have, however, provided unequivocal evidence that some of the glutamate taken up into astrocytes undergoes oxidative metabolism (for references, see McKenna, 2013; McKenna et al., 2016; Olsen & Sonnewald, 2015; Parpura et al., 2017; Schousboe, Scafidi, Bak, Waagepetersen, & McKenna, 2014; Sonnewald, 2014). Hence, the glutamate–glutamine cycle does not operate in a 1:1 stoichiometric fashion and therefore pyruvate carboxylation is an important reaction providing the means to maintain glutamate levels constant even when glutamate serves the role as an energy substrate.

2.6 | Activity of PC relative to the rate of glutamate cycling

If indeed, as originally suggested, the glutamate–glutamine cycle did operate in a stoichiometric fashion, the rate of the PC-catalyzed fixation of CO_2 should be negligible compared to that of the cycling rate. A number of studies have been performed aimed at elucidating this question (for references, see Table 1 and Öz, Okar, & Henry, 2012). According to these studies, the rate of the PC reaction varies between 6% and 50% of the rate of the cycle, i.e., a rather high degree of variation. However, it may be safe to conclude that the rate of pyruvate carboxylation is not negligible strongly underlining the fact that numerous studies as pointed out above have shown a high rate of glutamate oxidation. In a study using both ^{13}C-labeled glucose and ^{14}C-labeled bicarbonate instead of only [^{13}C]glucose to estimate the two rates (Öz et al., 2004), it was found that the rate of the PC reaction was around 30% of that of the cycle, i.e., close to the average of the abovementioned rates determined using [^{13}C]glucose as the only labeled substrate.

2.7 | Net synthesis and release of citrate from astrocytes

As discussed above, astrocytes are obligatory for the de novo synthesis of glutamine. Interestingly, it was shown using cultures of neurons and astrocytes and [^{13}C]glucose to label metabolites and subsequently NMR spectroscopy for analysis of the labeling patterns that cultured astrocytes but not cultured neurons exhibited a considerable release of [^{13}C]citrate to the incubation media (Sonnewald et al., 1991). Labeling of citrate was also observed in a similar ^{13}C labeling study using guinea pig brain slices by Badar-Goffer, Bachelard, and Morris (1990), but these authors could not show a release of citrate. Citrate release from neurons and astrocytes was subsequently determined in preparations of glutamatergic neurons (cerebellar granule cells) as well as in cerebellar astrocytes (Westergaard, Waagepetersen, Belhage, & Schousboe, 2017; Westergaard et al., 1994) and the release rate was almost 20 times higher in astrocytes compared to neurons. Interestingly, the release rate was similar to the activity of PC and the rate of CO_2 fixation in astrocytes (Kaufmann & Driscoll, 1992; Yu et al., 1983). Altogether, these findings underline

TABLE 1 Metabolic fluxes (μmol/min/g) in neurons and astrocytes measured in awake subjects using ^{13}C MRS

	PDH_n	PC	%PC	PDH_g	$\%PDH_g$	CMR_{glc}
Rat[a]	1–1.2	0.17 ± 0.05	10.4 ± 0.4	0.4 ± 0.06	23.5–18	1–1.5[c]
Human[b]	0.7 ± 0.2	0.06 ± 0.03	7	0.13 ± 0.04	15	0.4–0.45[c]

Note. PDH_n, Pyruvate dehydrogenase-mediated pyruvate flux in neurons (equals flux in neuronal TCA cycle); PC, Pyruvate carboxylase-mediated flux (astrocyte-specific); %PC, Pyruvate carboxylase-mediated flux as a percentage of total measured pyruvate metabolism; PDH_g, Pyruvate dehydrogenase-mediated pyruvate flux in glial cells (astrocytes); $\%PDH_g$, Pyruvate dehydrogenase-mediated pyruvate flux in astrocytes as a percent of the total measured pyruvate metabolism.

[a]Hertz (2011). [b]Hyder et al. (2006) Values for %PC and %PDH are calculated by the authors. [c]McKenna et al. (2012).

the importance of the PC-catalyzed reaction in astrocytes without which citrate could not be released in considerable amounts. As mentioned above, this is due to the fact that citrate like α-ketoglutarate is a TCA cycle constituent and therefore its release would lead to the depletion of the TCA cycle constituent if no replenishment would be possible. The importance of this citrate release from astrocytes in terms of brain function has been discussed by Westergaard, Banke, Wahl, Sonnewald, and Schousboe (1995), Westergaard et al. (2017) and a detailed outline of this is beyond this review.

2.8 | Balancing anaplerosis with cataplerosis

While the turnover rate of the TCA cycle is high reflecting the high energy consumption of the brain (McKenna et al., 2012), the concentrations of the individual intermediaries are quite low and normally kept rather constant. It is therefore conceivable that the anaplerotic process allowing a net synthesis of its constituents must be coupled to release from the tissue and a cataplerotic process leading to the exit of intermediates from the TCA cycle including the conversion of 4-carbon units to 3-carbon units. The role of anaplerotic reactions for cellular metabolism in the brain has been studied widely and is discussed above. It is, however, less well known how this process is coupled to cataplerosis. Moreover, it is also not completely clear which roles that both of these pathways play in the regulation of amino acid, glucose, and fatty acid homeostasis. In the brain constituting a closed system due to the blood–brain barrier, a linkage between anaplerosis and cataplerosis should be emphasized. There may be several candidate mechanisms for cataplerosis in the brain, one of which may be the loss of glutamine representing a possible way of counteracting anaplerosis. However, studies on glutamine release from brain have failed to show a substantial efflux (Dejong, Deutz, & Soeters, 1993).

Pyruvate recycling (Figure 2) represents a pathway for complete oxidation of glutamate and it is known to be active in the liver and has also been described in the brain by Cerdan, Kunnecke, and Seelig (1990). This recycling was originally believed to take place primarily in neurons and not in astrocytes because labeling patterns related to recycling were apparent in glutamate but not glutamine. Subsequent studies did, however, lead to the conclusion that this process is active in both astrocytes and neurons (Olsen & Sonnewald, 2015). The process of pyruvate recycling in the brain was additionally demonstrated in other animal studies (Haberg et al., 1998; Melo, Sonnewald, Touret, & Nehlig, 2006; Morken et al., 2014; Sonnewald, Therrien, & Butterworth, 1996). However, the process of pyruvate recycling could not be detected in rabbit brain or by in vivo MRS studies (Duarte, Lanz, & Gruetter, 2011; Lapidot & Gopher, 1994). The level of activity representing pyruvate recycling appears rather small and at present it does not appear conceivable that this process can match the activity of pyruvate carboxylation. Therefore, its physiological relevance has been debated and a logical explanation for the oxidation of glutamate to produce pyruvate would be an increase during hypoglycemia, a condition in which the pyruvate content is low. Experiments designed to prove this have, however, so far failed to do so (Bakken, White, Unsgard, Aasly, & Sonnewald, 1998).

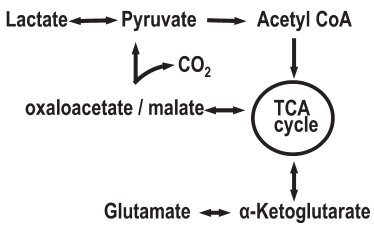

FIGURE 2 Schematic presentation of partial (lactate formation) and full (re-entry into the TCA cycle) pyruvate recycling. Abbreviation: TCA, tricarboxylic acid

It may be of interest in this context that lactate may represent a metabolite that may be able to leave the brain in large enough quantities to compensate for the constant anaplerosis by pyruvate carboxylation (Dienel, 2012). The hypothesis that a considerable fraction of cataplerosis in the brain may be brought about by exporting lactate generated from the intermediates of the TCA cycle into the blood stream and perivascular area was suggested by Sonnewald (2014). It was concluded that anaplerosis and cataplerosis must be tightly coupled and that glutamate might be the key compound connecting both, whereas lactate might serve as the "missing link." This hypothesis also shifts the generally accepted paradigm that lactate is exclusively derived from glycolysis to a possibility that it may originate from oxidation and might explain the phenomenon of "aerobic glycolysis" (Goyal, Hawrylycz, Miller, Snyder, & Raichle, 2014) under resting conditions. Results which support this hypothesis (Sonnewald, 2014) are the findings that lactate can be produced from the TCA cycle as shown in a number of experiments both in vivo (Hassel & Sonnewald, 1995) and in cell cultures (Alves et al., 2000; Amaral, Teixeira, Sonnewald, & Alves, 2011) and as mentioned above, that lactate can be exported from the brain (Dienel, 2012). In this context, it may also be of importance to note the connection between glutamate concentration and lactate formation. It was shown that partial pyruvate recycling, i.e., lactate formation from the TCA cycle (Figure 2), did increase with increasing extracellular glutamate concentrations in astrocytes (McKenna, Sonnewald, Huang, Stevenson, & Zielke, 1996). Combining the results from several publications (Olsen & Sonnewald, 2015), this coupling between the external glutamate concentration and lactate formation was confirmed and lactate production from the TCA cycle was shown to be more active in astrocytes than neurons, in line with the astrocytes' greater capacity for glutamate uptake.

Conducting conclusive experiments to clearly demonstrate cataplerotic pathways and how to couple them in a stoichiometric way to anaplerosis has been attempted but, thus far, without success. This is possibly due to the flexibility of the system and the many alternatives and, sometimes redundant, pathways that can be used.

2.9 | Ammonia homeostasis, glutamine synthetase, and alanine production: roles of PC and production of glycolytic pyruvate

Under physiological conditions, the ammonia concentration in the brain is maintained at very low levels due to the high activity of GS

and its low K_m value (0.2 mM) (Pamiljans, Krishnaswarmy, Dumville, & Meister, 1962). However, in a state of hepatic encephalopathy (HE) resulting from liver failure or disorders of urea cycle enzymes (Albrecht & Jones, 1999; Hazell & Butterworth, 1999), the ammonia concentration will rise drastically, a condition ultimately resulting in coma (Butterworth, 2002; Muñoz, 2008). During HE there is an increased synthesis of glutamine in an attempt to lower the ammonia level (Cooper, Mora, Cruz, & Gelbard, 1985; Hindfelt, Plum, & Duffy, 1977). This is associated with a stimulation of pyruvate carboxylation in order to compensate for an increased demand of α-ketoglutarate to provide the carbon skeleton for increased glutamine synthesis (Kanamatsu & Tsukada, 1999; Lapidot & Gopher, 1997; Zwingmann, Brand, Richter-Landsberg, & Leibfritz, 1998). Interestingly, inhibition of GS with methionine sulfoximine (MSO) has been shown to increase alanine production, shifting the metabolism of ammonia from fixation in glutamine to incorporation into alanine via the concerted action of glutamate dehydrogenase and alanine aminotransferase (Brusilow, Koehler, Traystman, & Cooper, 2010; Dadsetan et al., 2011, 2013; Fries et al., 2014; Leke et al., 2011). The latter series of processes lead to an enhanced glycolytic rate to provide pyruvate for alanine production (Dadsetan et al., 2011, 2013; Leke et al., 2011), which may underline the flexibility of the regulation of activities of metabolic pathways.

3 | CONCLUSIONS

One important outcome of the discovery that pyruvate carboxylation is an astrocytic, not neuronal process is the current concept that glutamatergic and GABAergic neurons are unable to perform a net synthesis of their neurotransmitters. In other words, there is an obligatory need for transfer of the precursor of these transmitters, i.e., glutamine from astrocytes to neurons. While the above is now generally accepted, it is still enigmatic to fully understand the mechanisms responsible for the balancing of anaplerosis, i.e., pyruvate carboxylation, with cataplerosis. This appears to be an experimental challenge since several pathways may be involved in glutamate homeostasis.

Another intriguing finding related to pyruvate carboxylation is the capacity for astrocytes to produce and release significant amounts of citrate. It was originally thought that citrate might represent a possible avenue for neurons to receive a TCA cycle constituent from surrounding astrocytes, but this appears unlikely to be the case (Westergaard et al., 2017). Another function of citrate may be related to its ability to chelate zinc ions thereby modulating the activity of NMDA receptors as suggested by Westergaard et al. (1995). Again, conclusive evidence for the functional significance of this still remains to be obtained.

Altogether, while 35 years of research related to the functional role of astrocytic pyruvate carboxylase activity has provided important new knowledge about the ability of astrocytes to influence in particular the glutamatergic activity, there may still be questions to be investigated such as how the balance between anaplerosis and cataplerosis is brought about.

CONFLICT OF INTEREST

The authors have no conflict of interest to be declared.

AUTHOR CONTRIBUTIONS

Review concept, A.S., H.S.W., U.S.; *Critical revision of manuscript*, A.S., U.S.; *Drafting of the manuscript*, A.S., H.S.W., U.S. All authors read and approved of the final version of the manuscript.

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