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REVIEW

Glutamate synthesis has to be matched by its degradation – where do all the carbons go?

Ursula Sonnewald

Department of Neuroscience, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

Abstract

The central process in energy production is the oxidation of acetyl-CoA to CO_2 by the tricarboxylic acid (TCA, Krebs, citric acid) cycle. However, this cycle functions also as a biosynthetic pathway from which intermediates leave to be converted primarily to glutamate, GABA, glutamine and aspartate and to a smaller extent to glucose derivatives and fatty acids in the brain. When TCA cycle ketoacids are removed, they must be replaced to permit the continued function of this essential pathway, by a process termed *anaplerosis.* Since the TCA cycle cannot act as a carbon sink, *anaplerosis* must be coupled with *cataplerosis*; the exit of intermediates from the TCA cycle. The role of anaplerotic reactions for cellular metabolism in the brain has been studied extensively. However, the coupling of this process with *cataplerosis* and the roles that both pathways play in the regulation of amino acid, glucose, and fatty acid homeostasis have not been emphasized. The concept of a linkage between *anaplerosis* and *cataplerosis* should be underscored, because the balance between these two processes is essential. The hypothesis that *cataplerosis* in the brain is achieved by exporting the lactate generated from the TCA cycle intermediates into the blood and perivascular area is presented. This shifts the generally accepted paradigm of lactate generation as simply derived from glycolysis to that of oxidation and might present an alternative explanation for aerobic glycolysis.

Keywords: anaplerosis, cataplerosis, glutamate oxidation, lactate.

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Anaplerosis

Sir Hans Kornberg (Kornberg 1966) coined the expression: *anaplerotic sequences* to describe a series of enzymatic reactions or pathways that replenish the pools of metabolic intermediates in the tricarboxylic acid (TCA) cycle. These intermediates are critical for the functioning of this cycle, the primary role of which is the oxidation of acetyl-CoA to carbon dioxide, energy production and, in brain, synthesis of precursors for the neurotransmitters: glutamate, GABA and aspartate. Even though pyruvate carboxylation has been shown to be the major anaplerotic reaction for the TCA cycle (Patel 1974), 4-and 5-carbon-containing intermediates enter the cycle during the catabolism of certain essential amino acids (Fig. 1), which cross the blood–brain barrier. Furthermore, 4-carbon units can enter after catabolism of the essential odd chain fatty acids (Fig. 1).

Anaplerosis from amino and odd chain fatty acids

The catabolism of the essential amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine proceeds via the TCA cycle and can be anaplerotic when the product is a TCA cycle intermediate other than acetyl CoA (Fig. 1). Since neurons (which lack pyruvate carboxylase) take up essential amino acids and odd chain fatty acids, it is conceivable that they can perform anaplerosis to a limited degree. Only few studies exist which shed light on the role of these compounds in anaplerosis. It has been shown that isoleucine has a low turn-over in rat brain and the authors concluded that this amino acid does not serve the role of providing metabolites pertinent to TCA cycle function (Bak *et al.* 2009). Similar results were obtained for threonine (Gaitonde 1975). Isoleucine and valine can, like the odd chain fatty acids, be converted into propionyl CoA that can enter the TCA cycle after conversion to succinyl CoA. Valine has been shown to be converted into

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Address correspondence and reprint requests to U. Sonnewald, Department of Neuroscience, Faculty of Medicine, Olav Kyrresgt. 3, N-7489 Trondheim, Norway. E-mail: ursula.sonnewald@ntnu.no

Abbreviations used: PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; TCA, tricarboxylic acid.

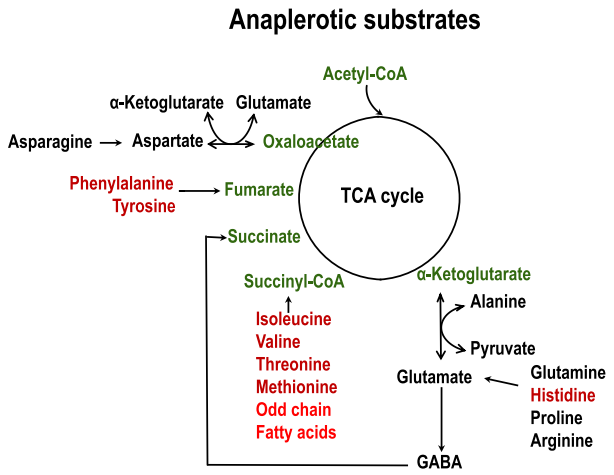

Fig. 1 Schematic representation of anaplerosis from amino and fatty acids, which cannot be synthesized by mammals and are ingested with the diet. Odd chain fatty acid derivatives and the amino acids: isoleucine, valine, threonine and methionine enter at the succinatyl CoA, phenylalanine and tyrosine at the fumarate, asparagine at the oxaloacetate and histidine at the α-ketoglutarate stage of the tricarboxylic acid (TCA) cycle. Names in green are TCA cycle intermediates; names in red anaplerotic substances, not made by the human body and thus transported into the brain; names in black are compounds that the human brain can make via pyruvate carboxylation.

glutamate, glutamine, lactate and other (non-TCA cycle related) metabolites in cultured astrocytes (Murin *et al.* 2009). Furthermore, the odd chain fatty acid heptanoate has been shown to have anaplerotic abilities (Borges and Sonnewald 2012; Hadera *et al.* 2013).

Pyruvate carboxylase

In brain, pyruvate carboxylase is the enzyme with a major responsibility for anaplerosis (Patel 1974). During development, pyruvate carboxylation is required since there is a high demand for the products: 4-carbon units like oxaloacetate and the five carbon unit α-ketoglutarate. They can provide the carbon skeleton for, or serve as precursors of the amino acid neurotransmitters: glutamate, GABA and aspartate. α-Ketoglutarate is a key compound since it can be converted into glutamate, which is the most abundant neurotransmitter and the precursor of GABA. However, pyruvate carboxylation and neurotransmitter synthesis and release do not occur in the same compartment. Carboxylation is restricted to astrocytes (Yu *et al.* 1983; Shank *et al.* 1985) whereas neurotransmitters are made in neurons. This dependence of neurons on astrocytes has long been acknowledged and is confirmed by the details of the glutamate-glutamine cycle which also encompasses a small amount of GABA which can be converted into glutamine after passing through the TCA cycle (Berl and Clarke 1983). It should be emphasized that these cycles do not perform *cataplerosis* or degradation of glutamate or GABA since the carbon backbones are returned to astrocytes that convert them into glutamine. It should be stressed that the TCA cycle cannot oxidize glutamate, glutamine, aspartate or GABA fully. Entry of these molecules in the form of α-ketoglutarate, oxaloacetate or succinate will lead to an increase in the amount of TCA cycle intermediates that cannot be reconciled with homeostasis. There is, thus, a serious problem with continuing anaplerosis in the adult brain. Theoretically, pyruvate carboxylation is not necessary at a time when concentrations of the neurotransmitters are constant or declining with age (Nilsen *et al.* 2012). One explanation for having turnover, i.e., separate mechanisms for synthesis and degradation, may be to have control of the concentration of a metabolite, which can be adjusted by either or both of these processes. The shorter the half life, the faster the change to a new steady-state level, i.e., degradation controls the time course of change of concentration, but not the synthesis rate. Whatever the reason, the fact is that there is anaplerosis and this would be detrimental if no pathway existed for the degradation of four and more carbon units. The present article is an attempt to present a solution to this problem.

De novo synthesis of the neuroactive non-essential amino acids glutamate, GABA, aspartate and glutamine requires anaplerosis, guaranteeing replenishment of the TCA cycle constituents which leave the cycle to be converted into these substances (McKenna *et al.* 2012). Several enzymes are suited for this function and, as mentioned above, in brain, pyruvate carboxylase (PC) is the most important (Patel 1974) and is located in astrocytes (Yu *et al.* 1983; Shank *et al.* 1985). For an in depth discussion of cellular localization of pyruvate carboxylation, please see Sonnewald and Rae (2010). Although malic enzyme or the combined action of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase can fix CO_2, they do not perform this function to a significant extent in the brain under physiological conditions (Patel 1974). Pyruvate carboxylation is particularly important in the developing brain for the production of TCA cycle intermediates and glutamine as well as the neurotransmitters glutamate, aspartate and GABA. The importance of pyruvate carboxylase underscores the finding that its absence causes metabolic acidosis and neurological impairment leading to psychomotor retardation and in many cases death in infancy (Schiff *et al.* 2006; Jitrapakdee *et al.* 2008). An excellent review on pyruvate carboxylase deficiency is by Marin-Valencia *et al.* (2010) and the clinical and biochemical response to anaplerotic diet therapy by Mochel *et al.* (2005).

Backflux

Anaplerosis is commonly measured by fixation of labelled CO_2 or by analysis of labeling patterns of glutamate derived from specifically-^{13}C-labelled glucose and ^{13}C NMR spectroscopy. Interpretation of results from studies

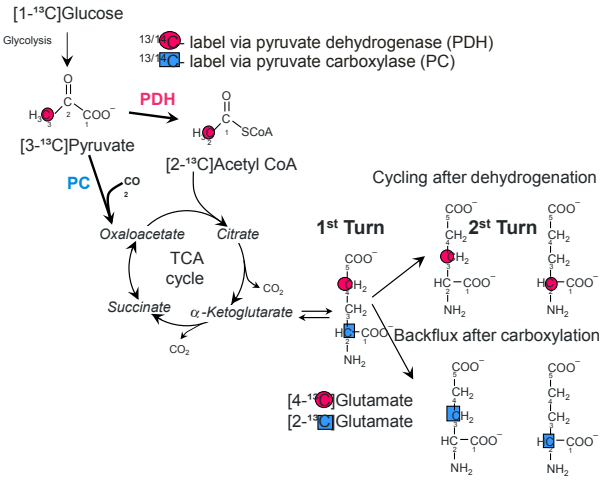

Fig. 2 Schematic representation of labelling form [1-^{13}C]glucose through the tricarboxylic acid (TCA cycle. Label from pyruvate dehydrogenase is shown as red filled circles and that from pyruvate carboxylase as blue filled squares. For simplicity only label in glutamate and not glutamine (identical to glutamate) and GABA (glutamate C-4 is GABA C-2 and Glutamate C-2 is GABA C-4) is shown. If the carbon atoms from α-ketoglutarate stay in the cycle beyond the first turn we get second turn metabolites with label either in the C-2 (glutamate, glutamine and the C-2 position for GABA) or C-3 positions (all three amino acids). If pyruvate is carboxylated to oxaloacetate which is directly condensed with acetyl CoA, then C-2 labelled glutamate (and glutamine and C-4 labeled GABA) is formed. However, backflux after pyruvate carboxylation (for details see Brekke *et al.* 2012) at the oxaloacetate stage to form the symmetrical fumarate followed by forward cycling will give rise to labelling of C-2 or C-3 glutamate (glutamine and C-4 or C-2 GABA) also. pyruvate carboxylase (PC), pyruvate carboxylation; PDH, pyruvate dehydrogenation.

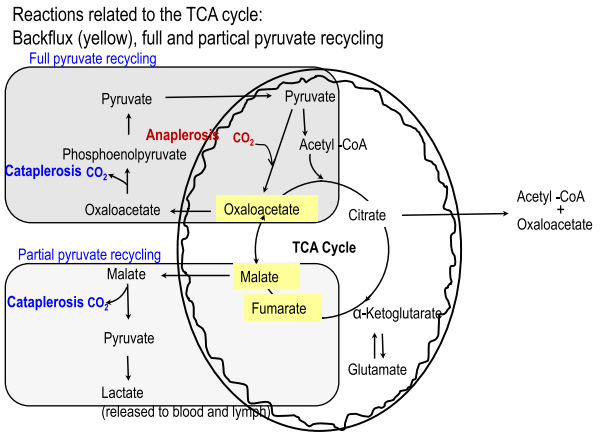

Fig. 3 Schematic representation of anaplerosis (in red) and cataplerosis (in blue) from pyruvate carboxylation. Pyruvate can enter the tricarboxylic acid (TCA) cycle via oxaloacetate (anaplerosis) and condenses with an acetyl CoA molecule to form citrate and eventually malate and oxaloacetate. Malate and/or oxaloacetate can leave the TCA cycle to be, after several steps, converted to lactate (cataplerosis). Full and partial pyruvate recycling are shown and backflux is indicated with yellow background.

on carboxylation using [1-^{13}C]glucose, as well as those using [1-^{14}C]glucose, is hampered by the fact that two different scenarios (Fig. 2) lead to the same labelling patterns in glutamate and glutamine: (i) labelled pyruvate is converted into labelled acetyl CoA by pyruvate dehydrogenase (PDH) activity and then merges with oxaloacetate forming citrate and then α-ketoglutarate, the carbon atoms of which then cycle beyond the first turn to form glutamate and glutamine from the second turn, (ii) backflux after pyruvate carboxylation at the oxaloacetate stage to form malate and then the symmetrical fumarate (Fig. 3) followed by forward cycling will give rise to identical labelling in glutamate and glutamine as mentioned in (i). The existence of this latter pathway was supported by mathematical models developed by Merle *et al.* (1996) to estimate this reversal of the TCA cycle when studying glutamine formation in cerebellar astrocytes. Furthermore, Gruetter *et al.* (2001) addressed the fact that neglecting backflux underestimates *in vivo* measurements of PC flux (Gruetter *et al.* 2001). The extent of backflux was calculated to be 27% using data from rats injected with [1-^{13}C]glucose and $H^{14}CO_3^-$ (Oz *et al.* 2004). Recently, we have shown substantial backflux using astrocyte cultures from cerebellum and neocortex and incubation with either [3–^{13}C]- or [2-^{13}C]glucose (Brekke *et al.* 2012). Applying our calculations to published data (Brekke *et al.* 2012), we demonstrated the existence of extensive backflux in cat, rat, mouse and human brain *in vivo*. Thus, backflux needs to be taken into account when calculating the magnitude of pyruvate carboxylation to allow for a precise evaluation of cerebral metabolism.

The magnitude of pyruvate carboxylation

As mentioned above, anaplerosis is important for net synthesis of glutamate, GABA and aspartate. In the immature rodent brain glutamate concentration is low at birth and increases till the end of the suckling period (Oja and Piha 1966; Tkáč *et al.* 2003). This increase in glutamate content necessitates pyruvate carboxylation and thus pyruvate carboxylase activity is relatively higher in 7-day-old rat pups compared to pyruvate dehydrogenase activity (Morken *et al.* 2013). However, even in the adult brain it has been shown that anaplerosis via pyruvate carboxylation occurs. Numerous reviews have shed light on the compartmentation and the extent of pyruvate carboxylation in adult rodents and humans (McKenna *et al.* 2012). With [1-^{13}C]glucose infusion, it could be shown that the C2 and C3 positions of glutamate and glutamine were not labelled identically in human brain, indicating some flux through PC (Gruetter *et al.* 1994). Mason *et al.* (2007)

were able to quantify this, showing in humans that pyruvate carboxylation accounted for 6% of glutamine synthesis (Mason *et al.* 2007). The data from a wide range of experiments have been compiled and the average % pyruvate carboxylase-mediated flux as a percentage of total measured pyruvate metabolism was 10, the range being: 4.9–12.5, with most values clustering around 10% (Hertz 2011). Correcting this for backflux, as addressed above, will lead to a higher value for pyruvate carboxylation under physiological conditions. It is possible to give some quantitative data: As illustrated in Fig. 4 (see below) astrocytic pyruvate metabolism accounts for about 37% (corrected for backflux of 27%; Oz *et al.* 2004) of the total, with 13% for PC and 24% for PDH (Qu *et al.*, 2000). The anaplerotic rate, since PDH must match PC, is 13% × 2 = 26%. Thus 26% of glucose is converted into a 5-carbon compound per time unit. Since 1/6 carbon unit (pyruvate to acetyl CoA) is lost at the PDH step during anaplerosis, 26−4% (calculated from: 26 × 1/6) gives an anaplerosis rate of 22% of glucose metabolized to make 5 carbon compounds.

Alterations in pyruvate carboxylation

Serres *et al.* (2008) found that carboxylation is connected to cerebral activity, as flux through pyruvate carboxylase is higher in the awake state, compared to deep phenobarbital anaesthesia. There are several reports in the literature of altered pyruvate carboxylase activity. Ischemia and traumatic brain injury are known to lower PC activity (Haberg and Sonnewald 2004; Haberg *et al.* 2006; Richards *et al.* 2007; Scafidi *et al.* 2009) and so does epilepsy (Smeland *et al.* 2013); whereas the ketogenic diet increased pyruvate carboxylation (Melø *et al.* 2006). It is also known that pyruvate carboxylase activity increases with increasing ammonia levels (Waelsch *et al.* 1964; Kanamatsu and Tsukada 1999; Dadsetan *et al.* 2011; Leke *et al.* 2011) linked to increased production of glutamine. Applying the quantitative approach started for anaplerosis the following

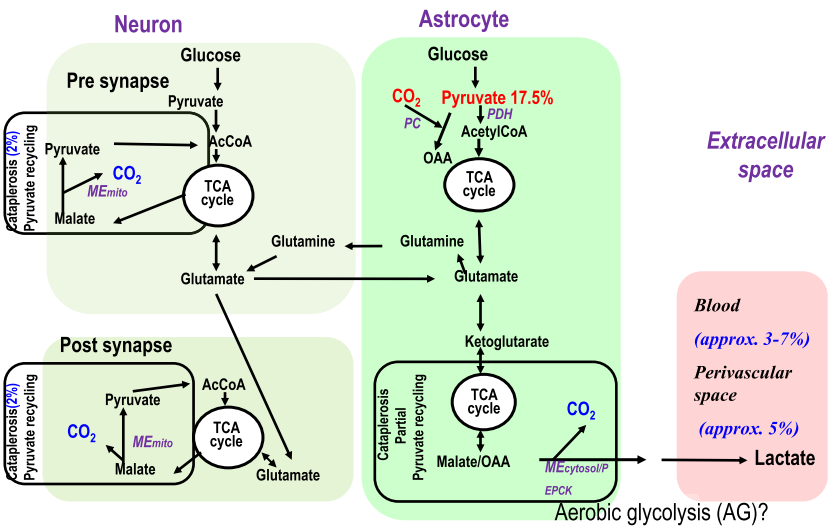

Fig. 4 Schematic representation of the unifying hypothesis for anaplerosis and cataplerosis indicating % of total glucose flux: red number represent anaplerosis, blue numbers cataplerosis. The calculations are as follows: astrocytic pyruvate metabolism accounts for about 37% [corrected for backflux of 27% Oz *et al.* (2004)] of total, with 13% for pyruvate carboxylase (PC) and 24% for PDH (Qu *et al.*, 2000). The anaplerotic rate, since PDH must match PC, is 13% × 2 = 26%. Thus 26% of glucose is converted to a 5-carbon compound per time unit. Since 1/6 carbon unit (pyruvate to acetyl CoA) is lost at the PDH step during anaplerosis, 26−4% (calculated from: 26 × 1/6) gives an anaplerosis rate of 22% of glucose metabolized to make 5 carbon compounds. For cataplerosis, re-entry of glutamate into the tricarboxylic acid (TCA) cycle starts with a 5-carbon compound, with 1 carbon atom lost in the TCA cycle and another lost in formation of trioses pyruvate/lactate, or 2/5 of this carbon pool is decarboxylated. So now the net excess of carbon may be represented by a matched steady state cataplerotic rate of 22% (or the anaplerosis rate) −9% (calculated from: 22% × 2/5), to give 12% of glucose flux that might be expected to be released from brain as lactate. In neurons and astrocytes, malate can be converted into pyruvate which can enter the TCA cycle after conversion into acetyl CoA (full pyruvate recycling, cataplerosis). In astrocytes that have cytosolic malic enzyme and phosphoenolpyruvate carboxykinase (PEPCK), malate and oxaloacetate can be converted into lactate (partial pyruvate recycling, cataplerosis) which can leave the brain via the blood or to the periventricular system. It has been shown that 3–7% of glucose passes from the brain to the blood as lactate and a significant amount enters the perivascular area and lymphatic drainage of lactate is thought to be considerable (set by the present author to the conservative value of 5% in Fig. 4, see below) (Dienel 2012a,b). Adding the 2 × 2% from pyruvate recycling in neurons to these numbers, the calculation for cataplerosis would be the following: (2 + 2) + (5 + 3–7). ME, malic enzyme; mito, mitochondrial; OAA, oxaloacetate, PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; TCA, tricarboxylic acid.

would be the case: For cataplerosis, re-entry of glutamate into the TCA cycle starts with a 5-carbon compound, with 1 carbon atom lost in the TCA cycle and another lost in the formation of trioses pyruvate/lactate, or 2/5 of this carbon pool is decarboxylated. So now the net excess of carbon may be represented by a matched steady-state cataplerotic rate of 22% (or the anaplerosis rate)−9% (calculated from: 22% × 2/5), to give 12% of glucose flux that might be expected to be released from brain as lactate. This means that the anaplerotic–catabolic mismatch might be on the order of 12%. This value would be higher when backflux is higher or/and the other amino and fatty acids shown in Fig. 1 contribute significantly.

Cataplerosis

Because the TCA cycle cannot fully oxidize 4- and 5-carbon compounds, these intermediates must be removed from the cycle by a process termed *cataplerosis*. Cataplerosis may be linked to biosynthetic processes such as amino acid synthesis, gluconeogenesis and fatty acid synthesis (Fig. 1). Cataplerosis can also occur via a partial pyruvate recycling pathway. Cataplerotic enzymes include phosphoenolpyruvate carboxykinase (PEPCK), malic enzyme, glutamate dehydrogenase, aspartate aminotransferase, and citrate lyase.

Full pyruvate recycling

Oxidative metabolism is high for glutamate in astrocytes (Hertz and Hertz 2003; McKenna 2013). For complete oxidative degradation of glutamate to CO_2, the carbon skeleton of the TCA cycle intermediate has to exit the TCA cycle in the form of malate or oxaloacetate, which can be converted into pyruvate, and subsequently re-enter the TCA cycle as acetyl-CoA generated from pyruvate (Figs 3 and 4). This is part of the so-called 'pyruvate recycling' pathway which has been reported in rat brain (Cerdan *et al.* 1990). Parts of the pyruvate recycling pathway have been detected in cultured astrocytes (Sonnewald *et al.* 1996; Waagepetersen *et al.* 2002) and neurons (Olstad *et al.* 2007; Amaral *et al.* 2011). High levels of pyruvate recycling from alternative substrates have been reported in developing brain (Scafidi *et al.* 2010) and it is conceivable that this process becomes more pronounced *in vivo* during hypoglycaemia. However, in cell culture experiments this could not be confirmed. In fact, the opposite was observed; pyruvate recycling was abolished during hypoglycaemia and aglycaemia in astrocytes (Bakken *et al.* 1998).

Lapidot *et al.* found no evidence for the existence of cerebral pyruvate recycling in rabbit brain (Lapidot and Gopher 1994). Hassel and Sonnewald (1995) found some lactate formation from [1,2-^{13}C]acetate, a process possible in astrocytes only and demonstrating the first part of pyruvate recycling; formation of pyruvate from TCA cycle intermediates (Hassel and Sonnewald 1995). Also Haberg *et al.* (1998) found pyruvate formation from [1,2-^{13}C]acetate, and additionally the re-entry of small amounts into the TCA cycle (Haberg *et al.* 1998). In conclusion, there is no strong indication that pyruvate recycling is a very active pathway for the removal of glutamate and thus cataplerosis. On the basis of the calculations in the present paper, it could possibly be around 2% of glucose flux.

Partial pyruvate recycling

Another possibility for removal of metabolites generated from pyruvate carboxylation is that four (or more) carbon units leave the brain. The most obvious candidate for this solution is glutamine. It has been shown in numerous papers that this compound can leave the brain via the blood–brain barrier and it is an important occurrence when ammonia concentration is high in the blood (Dejong *et al.* 1993). Nevertheless, when ammonia content is in the normal range, little glutamine leaves the brain (Dejong *et al.* 1993). However, one compound that does leave the brain is lactate (Madsen *et al.*, 1999; Dienel 2012a,b). But, lactate is obviously not a 4 or more carbon-containing compound and thus might not fit the description. To resolve this discrepancy, another look at the pyruvate recycling pathway is required. As pointed out above, it might not be feasible for the pyruvate generated from the TCA cycle intermediates to re-enter the cycle for 'full' pyruvate recycling. However, it might be possible for this pyruvate to be converted to lactate (Figs 3 and 4), and to leave the brain. The cells in which this could happen are astrocytes. It has been shown that malic enzyme in neurons is located in the mitochondria whereas that in astrocytes is located primarily in the cytosol (Kurz *et al.* 1993; McKenna *et al.* 1995, 2000). This might point towards the cytosol in astrocytes as the major compartment in which this lactate is generated. Indications for this are the formation of lactate from ^{13}C labelled acetate (metabolized in astrocytes only) (Hassel and Sonnewald 1995; Haberg *et al.* 1998) and the finding that extracellular glutamate is taken up into astrocytes and with increasing concentration gets converted to increasing amounts of lactate via the TCA cycle (Sonnewald *et al.* 1993; McKenna *et al.* 1996). Furthermore, lactate from TCA cycle intermediates is found in the medium, which shows that astrocytes release it (Sonnewald *et al.* 1993; McKenna *et al.* 1996). Furthermore, the connection between glutamate release from neurons and lactate formation in astrocytes via the TCA cycle could be the reason for the mismatch between ^{13}C enrichment in glucose and lactate. If all lactate is from glucose directly, the enrichment in lactate should reflect that of glucose. However, experiments in which rats are injected with [1-^{13}C]glucose this was not the case (Eyjolfsson *et al.* 2011). Lactate was approximately 10% less labelled than expected, fitting well with a cataplerotic production of lactate from vesicular glutamate which has a slower turnover

of ^{13}C label than the glutamate pool which is in direct equilibrium with the TCA cycle (Waagepetersen *et al.* 2005). It should also be mentioned that Kaufman and Driscoll (1992) have shown that pyruvate carboxylation in astrocytes increases with increased potassium concentration; however, no change was observed by Hassel and Sonnewald (2002).

Anaplerosis and cataplerosis balance

Can the amount of lactate released be adequate to compensate for the discrepancy of 12% between anaplerosis and cataplerosis? It has been shown that 3–7% of glucose passes from the brain to the blood as lactate and a significant amount enters the perivascular area and lymphatic drainage of lactate is thought to be considerable (set by the present author to the conservative value of 5% in Fig. 4, see below) (Dienel 2012a,b). Adding the $2 \times 2\%$ from pyruvate recycling in neurons to these numbers the calculation for cataplerosis would be the following: (2 + 2)+(5 + 3–7).

Lactate production is usually coupled to anaerobic glycolysis but has also been shown to take place in the presence of oxygen. This is called the Warburg effect traditionally or more recently aerobic glycolysis and is described in very recent papers analysing glucose metabolism in humans and primates respectively (Bauernfeind *et al.* 2014; Goyal *et al.* 2014). It is intriguing to hypothesise that this lactate production is not from glycolysis but from the TCA cycle. The suggested amount of aerobic glycolysis was 10% of glucose consumption (Goyal *et al.* 2014) and would fit well with the numbers in this paper. Another point related to the above work is the usual finding that the CMRO2/CMRglc ratio is < 6 in resting brain, indicating some non-oxidative metabolism that is often ascribed to lactate production, but involves all non-oxidative pathways of glucose metabolism (e.g. glycogen and amino acid synthesis). This means that the discussion related to aerobic glycolysis and the anabolic-catabolic mismatch needs some integration so that the lactate release via glutamate catabolism could also explain the lower-than-theoretical CMRO2/CMRglc by another mechanism and set of pathways, separate from the simple idea of glucose to lactate and lactate release.

It is important to emphasize that the present hypothesis applies to resting steady state conditions with relatively low amounts of lactate released from brain, and that during brain activation or under pathophysiological conditions when glycolysis is strongly upregulated aerobic glycolysis may reflect mainly glucose to lactate and release of glucose-derived lactate from brain. More experiments are obviously needed to verify the present hypothesis. It is known that labelled and unlabelled lactate is released from brain, but the fractional enrichment of the released lactate is not known since it mixes rapidly with systemically labelled lactate. Compartmentation of lactate metabolism is another problem that has to be addressed in more detail in the future.

Conclusion

Anaplerosis and cataplerosis are tightly coupled and glutamate appears to be the key compound connecting them, whereas lactate is the 'missing link'. Its efflux compensates for the constant anaplerosis by pyruvate carboxylation (Figs 3 and 4). This hypothesis shifts the generally accepted paradigm of lactate generation as simply derived from glycolysis to that of oxidation and might be an explanation for the phenomenon of 'aerobic glycolysis'.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

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