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Carboxylation and Anaplerosis in Neurons and Glia

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Abstract

Anaplerosis, or *de novo* formation of intermediates of the tricarboxylic acid (TCA) cycle, compensates for losses of TCA cycle intermediates, especially α-ketoglutarate, from brain cells. Loss of α-ketoglutarate occurs through release of glutamate and GABA from neurons and through export of glutamine from glia, because these amino acids are α-ketoglutarate derivatives. Anaplerosis in the brain may involve four different carboxylating enzymes: malic enzyme, phosphoenopyruvate carboxykinase (PEPCK), propionyl~CoA carboxylase, and pyruvate carboxylase. Anaplerotic carboxylation was for many years thought to occur only in glia through pyruvate carboxylase; therefore, loss of transmitter glutamate and GABA from neurons was thought to be compensated by uptake of glutamine from glia. Recently, however, anaplerotic pyruvate carboxylation was demonstrated in glutamatergic neurons, meaning that these neurons to some extent can maintain transmitter synthesis independently of glutamine. Malic enzyme, which may carboxylate pyruvate, was recently detected in neurons. The available data suggest that neuronal and glial pyruvate carboxylation could operate at as much as 30% and 40–60% of the TCA cycle rate, respectively. Cerebral carboxylation reactions are probably balanced by decarboxylation reactions,, because cerebral CO_2 formation equals O_2 consumption. The finding of pyruvate carboxylation in neurons entails a major revision of the concept of the glutamine cycle.

Index Entries: Anaplerosis; pyruvate carboxylation; malic enzyme; tricarboxylic acid cycle; glutamine; GABA; glutamine; neuron; glia.

Introduction

Recently carboxylation of pyruvate to malate or oxaloacetate was shown to occur in neurons *(1,2).* The active enzyme may have been malic enzyme, which has been detected in neurons *(1,3–5).* The finding of neuronal pyruvate carboxylation means that neurons, at least to some extent, may support their formation of transmitter glutamate and GABA without making use of glutamine imported from glia. This conclusion has consequences for the concept of a metabolic interplay between neurons and glia, the glutamine cycle, which implies that transmitter glutamate and GABA are formed from glial glutamine. The theoretical basis of the glutamine cycle has been the notion that cerebral pyruvate carboxylation

only occurs in glia *(6,7).* Now that pyruvate carboxylation has been demonstrated in neurons it may be useful to re-evaluate the role of glutamine in glutamatergic and GABAergic neurotransmission.

Carboxylation is the addition of CO_2 or bicarbonate to biological molecules to yield carboxylic groups:

$$R + CO_2 \text{ or } HCO_3^- \rightarrow R\text{–}COO^-$$

The brain has a large net production of CO_2, equivalent to the consumption of O_2 (for review, *see* ref. *8*). Therefore, cerebral carboxylation must by and large be balanced by complementary decarboxylation reactions. Because there is intercellular and intracellular transfer of products of carboxylation, it follows that carboxylation and the corresponding decarboxylation reactions may occur in different cell types or cellular compartments.

Anaplerosis is the *de novo* synthesis of intermediates of the TCA cycle to replenish losses of intermediates. Carboxylation and anaplerosis are only partially overlapping phenomena: carboxylation occurs in both anaplerotic and nonanaplerotic pathways, and anaplerosis may occur through pathways that do not involve carboxylation, such as degradation of some of the glucogenic amino acids. The brain has a restricted entry of amino acids across the blood-brain barrier (BBB) and the influx of large neutral amino acids, which cross the BBB easily and which may enter anaplerotic pathways, has been estimated at 28 nmol/g tissue × min^{-1} in the rat *(9),* or only ~3% of the cerebral uptake of glucose in awake animals *(10).* Therefore, carboxylation, mainly of pyruvate, is considered the predominant anaplerotic pathway in the intact brain. In cultured cells, which are grown in amino acid-rich media, glucogenic amino acids may contribute more to anaplerosis than they do in vivo. In Eagle's minimum essential medium, which is often used for culturing neurons and astrocytes, the total concentration of amino acids that may yield TCA cycle intermediates, is 4.1 m*M* (Table 1), whereas the concentration of glucose is 5.5 m*M (11).* In the various formulations of Dulbecco's modified Eagle medium (DMEM) the concentration of these amino acids, which all enter both neurons and astrocytes *(12),* may be considerably higher. The metabolism of glucogenic amino acids is probably why glucose contributes so little to the formation of glutamine in cultured astrocytes *(13).*

The issue of anaplerotic carboxylation in the CNS arose in 1951 when Crane and Ball *(14)* found CO_2 fixation in isolated retina, both through an ATP-dependent reaction and through and NADPH-requiring and ATP-independent reaction that could correspond to the reactions catalyzed by pyruvate carboxylase and malic enzyme, respectively. In 1953, Moldave et al. *(15)* reported CO_2 fixation in mouse brain and labeling of aspartate and glutamate from $H^{14}CO_3^-$. Since the early 1960s, the question of the cellular localization of anaplerotic carboxylation in the brain has been a recurring issue. Cheng *(16)* gives a review of the first 20 years of research into cerebral carboxylation.

An Overview of Carboxylation Reactions

Because carboxylation and anaplerosis often have been used synonymously in the literature, it may be useful to review the field of carboxylation. Carboxylation reactions may be roughly divided into three categories: anabolic, catabolic, and protein carboxylation (Table 2).

Anabolic Carboxylation

De novo formation of malate or oxaloacetate may occur through three different carboxylation reactions:

1. Carboxylation of pyruvate to malate by malic enzyme; this reversible enzyme has two isoforms, one cytosolic, the other mitochondrial. The reaction depends on NADPH and Mg^{2+} or Mn^{2+}: Pyruvate + CO_2 + NADPH + $H^+ \rightarrow$ malate + $NADP^+$.

   Both the mitochondrial and the cytosolic isozymes have been detected in astrocytes

Table 1
Amino Acids That are Degraded to TCA Cycle Intermediates and Their Concentration in Eagle's Minimum Essential Medium and in Rat Plasma[a]

Amino acid	TCA cycle intermediate	Eagle's MEM (mM)	Plasma (μM)
Arginine	α-Ketoglutarate	0.6	80
Glutamine		2	600
Histidine		0.2	50
Proline		0.1[b]	
Glutamate			
Isoleucine	Succinate	0.4	70
Methionine		0.1	40
Valine		0.4	140
GABA			
Phenylalanine	Fumarate	0.2	50
Tyrosine		0.2	90
Asparagine	Oxaloacetate	0.1[b]	
Aspartate			

[a] The concentrations of amino acids in Eagle's minimum essential medium (MEM) are calculated from the values given by Life Technologies *(11);* the plasma levels are from Pardridge *(114).* Observe that there is probably a net efflux of glutamine from the brain to the circulation in vivo *(69).*

[b] Only present in some formulations. Plasma concentrations for amino acids that presumably do not enter the brain to any significant extent are not given.

Table 2
Enzymes that Catalyze Carboxylation Reactions

Anabolic carboxylation
Malic enzymes
PEPCK
Pyruvate carboxylase
NAD(P) isocitrate dehydrogenase
Acetyl~CoA carboxylase
Glycine synthase
Carbamoyl-phosphate synthase II
Phosphoribosylamino-imidazole carboxylase

Catabolic carboxylation
Propionyl~CoA carboxylase
β-Methylcrotonyl~CoA carboxylase
Carbamoylphosphate synthetase I (not in brain)

Protein carboxylation
γ-Glutamyl carboxylase

(17,18) and neurons *(3–5)* with the use of immunocytochemistry and enzymatic methods. Malic enzyme has long been considered an anaplerotic enzyme in heart *(19),* but brain researchers have so far tended to view it as a decarboxylating enzyme *(3,5).*

2. Carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase; this enzyme is mitochondrial, biotin-dependent, activated by acetyl~CoA, and it requires ATP: Pyruvate + HCO_3^- + ATP → oxaloacetate + ADP.

Pyruvate carboxylase was shown by biochemical methods to be expressed in cultured astrocytes, but not in cultured neurons *(20–22),* and immunocytochemical detection confirmed these observations *(23).*

3. Reversal of the phosphoenolpyruvate carboxykinase (PEPCK) reaction would lead to carboxylation of phosphoenolpyruvate, an intermediate of glycolysis, to oxaloacetate. The enzyme, which in the brain is predominantly mitochondrial, has been detected in primary cultures of neurons and astrocytes, and in adult brain *(4,24–27).* Its

carboxylating activity has probably been shown in intact liver cells *(28)*, and is the basis of a useful enzyme assay *(29)*, but PEPCK is generally considered to operate in the decarboxylating direction.

Anabolic Carboxylation Reactions that are not Anaplerotic

Fatty acids are synthesized from acetyl~CoA and malonyl~CoA by fatty acid synthase; malonyl~CoA is formed by carboxylation of acetyl~CoA by the biotin-dependent enzyme acetyl~CoA carboxylase. Formation of the 16-carbon palmitic acid requires, in addition to one molecule of acetyl~CoA, 7 malonyl groups, each of which is decarboxylated as they enter the growing carbon chain: the HCO_3^- that was fixed, is lost as CO_2. Fatty acid synthase was recently detected in neurons by immunocytochemistry *(30)*, strongly suggesting that acetyl~CoA carboxylase is also expressed in neurons. Previously acetyl-CoA carboxylase and fatty and synthase have been demonstrated in oligodendrocytes *(31)*.

Synthesis of nucleotides involves carboxylation. The pyrimidines are made from aspartate and carbamoylphosphate; the latter is formed from CO_2 and the amide of glutamine. This reaction is catalyzed by the cytosolic enzyme carbamoyl-phosphate synthase II:

$$CO_2 + \text{glutamine} + 2\ ATP + H_2O \rightarrow \text{carbamoylphosphate} + \text{glutamate} + 2\ ADP + P_i.$$

Carbamoyl transferase II is expressed by astrocytes and neurons as shown by *in situ* hybridization and immunocytochemistry *(31–33)*. Early biochemical studies showed that uridine formation was low in cerebellar slices from newborn rats when maintained in culture for 10–12 d *(34)*; this finding suggested that pyrimidine synthesis to some degree depends on recycling (the salvage pathway) rather than complete degradation and *de novo* synthesis.

In purine synthesis an imidazole ring is formed with 5-phospho-α-D-ribose-5-phosphate (PRPP) as a starting point. To the imidazole ring a molecule of CO_2 (the C_6) is added by phosphoribosylaminoimidazole carboxylase, then an amino group from aspartate, and a formyl group are added. Purine synthesis is low in adult rat brain *(35)*, and brain purines are mainly derived from the circulation *(36)*.

Glycine may be formed from serine or through a carboxylation reaction that is catalyzed by the reversible mitochondrial enzyme glycine synthase (glycine-cleavage enzyme):

$$CO_2 + NH_3 + \text{methylenetetrahydrofolate} + NADH + H^+ \rightarrow \text{glycine} + \text{tetrahydrofolate} + NAD.$$

The enzyme is active in brain *(37)* and has been detected in astrocytes *(38)*.

The isocitrate dehydrogenase reaction may be reversed so that α-ketoglutarate is carboxylated to another TCA cycle intermediate, isocitrate. The reaction may take place in both mitochondria, by NAD-dependent or NADP-dependent isocitrate dehydrogenase, and cytosol, by NADP-dependent isocitrate dehydrogenase:

$$\alpha\text{-ketoglutarate} + CO_2 + NAD(P)H + H^+ \rightarrow \text{isocitrate} + NAD(P)^+$$

This reaction is not anaplerotic, because it does not increase the total pool of TCA cycle intermediates. If isocitrate is metabolized through the TCA cycle, the fixed CO_2 is rapidly lost again, but isocitrate may be converted to citrate through reversal of aconitase, and citrate may be cleaved to acetyl~CoA and oxaloacetate. Through such a pathway, which has been demonstrated in striatal slices incubated with [1-^{14}C]glutamate *(39)*, the fixed CO_2 may be retained in the TCA cycle for a long time. Reversal of the isocitrate dehydrogenase reaction has also been shown in cortical slices (*25*) and in brain homogenates *(40)*.

Catabolic Carboxylation

Anaplerotic carboxylation also occurs when propionyl~CoA is carboxylated to methylmalonyl~CoA at the expense of ATP by the biotin-dependent enzyme propionyl~CoA carboxylase; methylmalonyl~CoA is converted to succinyl~CoA which enters the TCA

cycle and becomes converted to succinate. Propionyl~CoA is formed during the metabolism of isoleucine, methionine, propionate, and valerate. Propionate and valerate are normally present in serum at low μ*M* concentrations *(41).* Degradation of longer odd-numbered fatty acids also leads to formation of propionyl~CoA, but these are rare in terrestrial animals. Propionyl~CoA carboxylase has been detected in whole brain *(42)* and in cultured astrocytes *(43).* Propionyl~CoA carboxylase does not seem to have been investigated in neurons, but since cultured neurons degrade isoleucine to CO_2 *(44),* they presumably express this enzyme.

Leucine degradation leads to formation of β-methylcrotonyl~CoA which is carboxylated to β-methylglutaconyl~CoA by the biotin-dependent enzyme β-methylcrotonyl~CoA carboxylase at the expense of ATP; the final products of leucine degradation are acetyl~CoA and acetoacetate; therefore, leucine is not an anaplerotic substrate. β-Methylcrotonyl~CoA carboxylase has been detected in both cultured neurons and astrocytes *(43,45).*

In the urea cycle arginine is cleaved to urea and ornithine by arginase. Regeneration of arginine includes a carboxylation reaction to yield carbamoylphosphate (catalyzed by carbamoylphosphate synthetase I) which reacts with ornithine to yield citrulline. Citrulline reacts with aspartate to argininosuccinate, which is cleaved to arginine and fumarate. Carbamoylphosphate synthetase I is not expressed in brain, although arginase is *(46,47).* The urea cycle is mentioned here, because arginine is important in the brain as the precursor for nitric oxide (NO). However, in the "nitric oxide cycle" arginine loses only NO and no carbon; therefore, the NO cycle does not involve carboxylation.

Protein Carboxylation

Protein carboxylation is a post-translational modification of proteins that occurs in the rough endoplasmatic reticulum and Golgi apparatus, catalyzed by vitamin K-dependent γ-glutamyl-carboxylase. The substrate of this enzyme is glutamyl residues that become carboxylated in the gamma position (the C_4); thus, this carbon, which already binds one carboxylic group, binds a second carboxylic group. The two, negatively charged, carboxylic groups may bind Ca^{2+} or other divalent cations. Protein carboxylation depends on vitamin K, which is oxidized to an epoxide in the reaction. The vitamin K antagonist warfarin inhibits the reduction of oxidized vitamin K by vitamin K epoxide reductase, thereby blocking protein carboxylation (for review, see ref. *48*).

γ-Glutamyl carboxylation was first detected in proteins of coagulation *(49).* Factors II (prothrombin), VII, IX, and X depend on carboxylation to achieve pro-coagulant activity, and protein C and protein S depend on carboxylation to regulate coagulability. In bone osteocalcin and matrix gla protein, two proteins involved in bone mineralization, are γ-glutamyl-carboxylated *(50).* Gas6 (growth arrest-specific) protein, which is involved in regulation of cell proliferation through activation of a tyrosine kinase receptor, also requires γ-glutamyl carboxylation for biological activity *(51,52).* γ-Glutamyl-carboxylase is expressed in the brain *(53),* and gas6 is expressed in neurons in the CNS both *in utero* and in the adult *(54).* A proline-rich γ-glutamyl-carboxylated protein of 23kD has been found in the spinal cord *(55),* but nothing has so far been published on its function. Warfarin exposure during pregnancy leads to severe disturbance of brain (and bone) development in human fetuses (e.g., ref. *56*), but it is not yet clear if this is due to inhibition of γ-glutamyl-carboxylation in the brain itself, or if it results from hemorrhages caused by a defective clotting mechanism. A method for immunopurification of proteins containing γ-carboxyglutamyl residues *(57)* may help in the detection of other γ-glutamyl-carboxylated proteins in the CNS. The possibility that such proteins could act as calcium sensors is attractive.

Several points emerge from the above. First, carboxylation reactions, both anaplerotic and

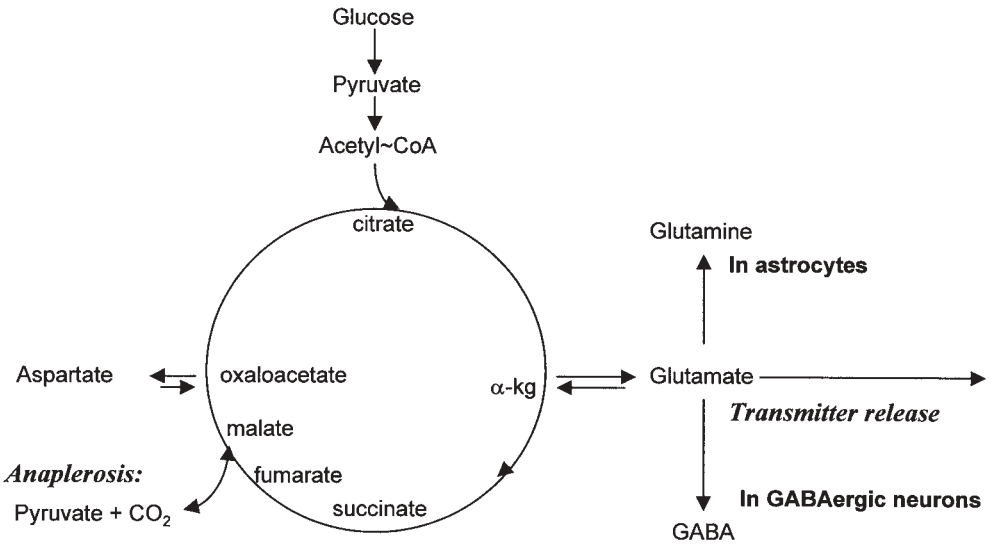

Fig. 1. Simplified scheme of the TCA cycle and the anaplerotic carboxylation that compensates for the loss of α-ketoglutarate inherent in the release of transmitter glutamate and GABA and glutamine.

nonanaplerotic, may occur in neurons and glia alike. Second, anaplerotic carboxylation is more than carboxylation of pyruvate; it includes carboxylation of propionyl~CoA and phosphoenolpyruvate. Third, detection of a carboxylating pathway through fixation of $H^{14}CO_3^-$, must involve identification of the labeled products. Simple measurement of acid-stable radioactivity may detect a sum of carboxylation reactions. It is also worth noticing that with the use of $H^{14}CO_3^-$ and subsequent detection of radiolabeling of amino acids [a common method of investigation *(1,39,58)*] aspartate will become better labeled than glutamate and glutamine. Aspartate may become labeled from $H^{14}CO_3^-$ both through carboxylation of pyruvate, phosphoenolpyruvate, and propionyl~CoA; the two former reactions yield malate or oxaloacetate, the latter succinate. Therefore, preferential radiolabeling of aspartate from $H^{14}CO_3^-$ does not necessarily reflect carboxylation of pyruvate.

It is difficult on the basis of existing data to say which carboxylating pathway contributes more to anaplerosis. The measured in vitro activities of the various enzymes differ markedly from one study to another *(1,2,4,24,25,40),* and there is a lack of good enzyme inhibitors. With respect to malic enzyme, the inhibitors bromopyruvate and hydroxymalonate block the decarboxylation reaction only.

Why Do Brain Cells Need Anaplerosis?

Anaplerosis is needed to replenish losses of TCA-cycle intermediates. Neurons lose TCA cycle intermediates during glutamatergic and GABAergic neurotransmission (Fig. 1). Glutamate and GABA are formed from the TCA cycle intermediate α-ketoglutarate. After release from nerve terminals, glutamate and GABA are removed from the synaptic cleft by uptake into astrocytes and neurons *(59,60).* Studies on the effect of immunoprecipitation of the glial glutamate transporter EAAT2 (GLT) suggest that in the forebrain most of transmitter glutamate is taken up by astrocytes *(61).* GABA transporters are also expressed in both

astrocytes and neurons *(62,63),* and radiolabeling studies suggest that in some brain structures, e.g., the cerebral cortex, the astrocytic uptake of GABA predominates over the neuronal uptake *(64),* whereas in others, e.g., the striatum, the neuronal uptake is quantitatively most important *(65).* Glial cells lose α-ketoglutarate through export of glutamine. It has been calculated that ~60% of astrocytic TCA cycle intermediates are continuously lost, mostly in the form of glutamine *(13,66).* Glutamine formation is a main pathway for inactivation of ammonia in the brain *(67,68),* and there is a net export of glutamine, ~10 nmol/g × min^{-1}, from the brain to the circulation *(69).* Glutamine is also exported from glia to neurons, where it is used as an energy substrate *(66,70,71)* and as a precursor for glutamate and GABA *(72–75).* Both neurons and astrocytes may lose TCA-cycle intermediates through decarboxylation of malate by malic enzyme, the reversal of the pyruvate carboxylation reaction. This reaction leads to formation of NADPH, which is used in a number of biosynthetic pathways and for reduction of oxidized glutathione. Glial cells, and to a lesser extent neurons, may also lose TCA cycle intermediates through export of citrate to the extracellular fluid *(76–78).*

A net loss of TCA cycle intermediates would reduce the oxidative capacity of the cell. This is due to the cycle nature of oxidative energy metabolism (*see* Fig. 1): a net loss of α-ketoglutarate, malate, or citrate will deplete the TCA cycle of oxaloacetate and reduce the ability of the cells to form citrate. A reduction in TCA-cycle activity would quickly lead to depletion of ATP and loss of membrane potentials, because the average half-life of the 3 m*M* of ATP in the brain is 3 seconds *(79).*

Glial cells (or the "small metabolic compartment") have long been known to possess anaplerotic carboxylating activity. Waelsch et al. *(58)* showed that intravenous infusion of radiolabeled bicarbonate labeled glutamine somewhat more avidly than glutamate, a sign that carboxylation occurred (at least in part) in the glutamine-synthetizing compartment, which was later identified as glia *(80,81).* The detection of the enzyme pyruvate carboxylase in astrocytes *(20–23)* confirmed the carboxylating capacity of glia. Therefore, glia could in theory compensate for losses of α-ketoglutarate caused by glutamine export, although it has never been rigorously shown that pyruvate carboxylase is the pyruvate-carboxylating enzyme that supports the glutamine export. In addition to the anaplerotic reactions mentioned in previous sections, glial anaplerosis includes metabolism of transmitter glutamate and GABA. Glutamate may be converted to α-ketoglutarate and enter the glial TCA cycle, or it may be converted directly into glutamine *(82,83)* by the glia-specific enzyme glutamine synthase *(80,81).* GABA, on the other hand, is degraded to succinyl~CoA and succinate, and it is therefore necessarily an anaplerotic substrate for glia, whereas glutamate may or may not serve glial anaplerosis.

Neuronal Pyruvate Carboxylation

Pyruvate carboxylation has until now not been considered a neuronal phenomenon. Instead, neurons have been thought to cover their anaplerotic needs by uptake of glutamine. One is struck by the beauty and simplicity of a model that depicts a flux of transmitter glutamate and GABA from neurons to glia and a compensatory transfer of glutamine in the opposite direction. However, Hertz *(84)* reasoned that neuronal uptake of glutamine could not fully account for the formation of transmitter amino acids, a warning that the model might be too simplistic. Further, although glutamine was shown in vitro to be a good precursor for releasable glutamate *(72,73)* and GABA *(74),* this was much more difficult to show in vivo (*85;* for review, *see* ref. *86*). In fact, injection of radiolabeled glutamine into brain leads mostly to formation of $^{14}CO_2$, not so much to formation of transmitter amino acids *(71).* It has also been shown that glutaminase, the neuronal enzyme that converts glutamine into glutamate, is not expressed in all glutamatergic

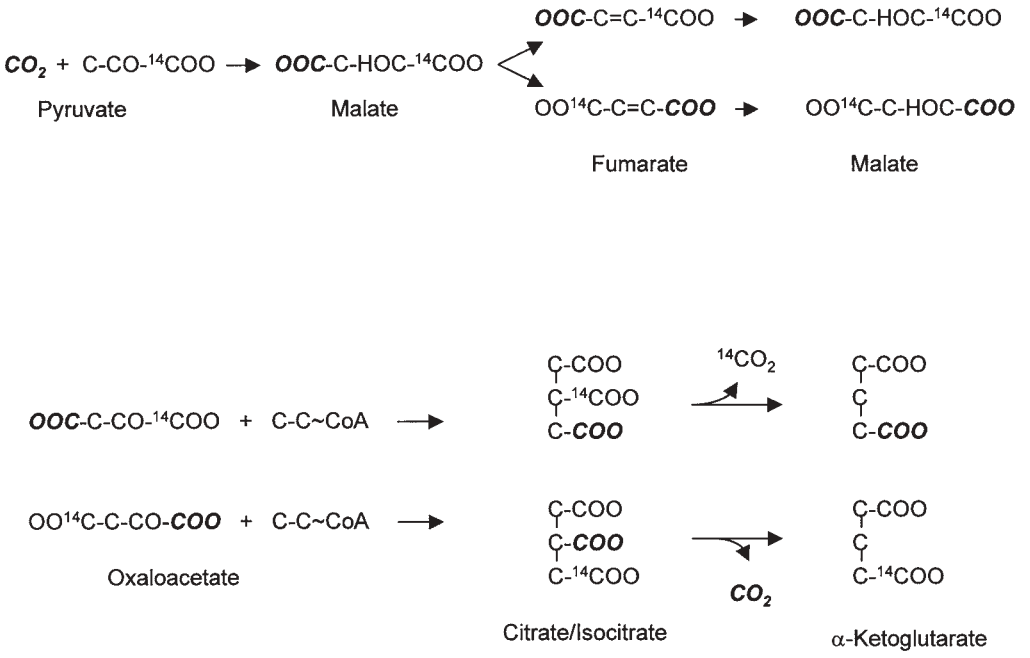

Fig. 2. Labeling of TCA cycle intermediates through carboxylation. [1–^{14}C]Pyruvate is carboxylated to malate. This malate must equilibrate with fumarate for the label to reach α-ketoglutarate (and hence glutamate and glutamine, which become labeled in the C_1), otherwise the label is lost as CO_2 in the isocitrate dehydrogenase reaction. Note that when [1–^{14}C]pyruvate is metabolized to acetyl~CoA through pyruvate dehydrogenase, the label is lost as $^{14}CO_2$. When radiolabeled CO_2 or HCO_3 is the precursor (shown in italics), equilibration of malate with fumarate leads to loss of labeling the isocitrate dehydrogenase reaction. When glutamate is decarboxylated to GABA, the labeled carboxylic group in glutamate is lost as $^{14}CO_2$.

and GABAergic neurons *(87–90).* Therefore, one would expect glutamatergic and GABAergic neurons, or at least subpopulations of such neurons, to be able to perform anaplerotic reactions.

Evidence for neuronal pyruvate carboxylation has been found in vitro and in vivo, often with the use of pyruvate labeled in the carboxylic group, [1-^{14}C]pyruvate (CH_3-CO-$^{14}COO^-$). When [1-^{14}C]pyruvate enters the TCA cycle through pyruvate dehydrogenase and is converted to acetyl~CoA, the label is lost as $^{14}CO_2$, but when the pyruvate is carboxylated, the label is retained in the carbon chain (Fig. 2). Cheng and Nakamura *(39)* found that incubation of brain slices with [1-^{14}C]pyruvate gave a higher specific activity in glutamate than in glutamine, reflecting that carboxylation occurred, at least in part, in cells that lacked glutamine synthetase, i.e., neurons *(80,81).* Hassel and Bråthe *(1)* found that glutamatergic cerebellar granule cells converted both [1-^{14}C]pyruvate and $H^{14}CO_3^-$ into glutamate and aspartate. Further, when the TCA cycle was blocked with 3-nitropropionic acid, an inhibitor of succinate dehydrogenase, the intracellular level of aspartate fell by 60%; this reduction could be prevented by addition of pyruvate at 20 m*M*, because pyruvate carboxylation was still operative. In vivo Van den Berg *(91)* found that intraperitoneal injection of [3,4-^{14}C]glucose, which is converted into [1-^{14}C]pyruvate through glycolysis, gave a higher specific activity in glutamate than in glutamine, and Hassel and Bråthe *(1)* found the same when [1-^{14}C]pyruvate or $H^{14}CO_3^-$ was

injected into rat striatum. These findings indicate that pyruvate carboxylation in the intact brain partly occurs in cells that do not express glutamine synthetase, i.e., neurons *(80,81).* Had pyruvate carboxylation occurred only in glia, then glutamine would have had the higher specific activity (for a more detailed explanation, *see* Section on Three Sources of Error in Isotopic Labeling Studies of Anaplerotic Carboxylation).

Neuronal anaplerotic carboxylation has an obvious function in compensating for the cellular loss of transmitter glutamate and GABA. More generally, anaplerotic carboxylation may strengthen the energy-producing capacity of the cells during increased energy demand by making more di- and tricarboxylic acids available for the dehydrogenases of the TCA cycle. One may envisage that carboxylation occurs during neuronal activation when membrane depolarization increases the demand for ATP for the re-establishment of ion gradients. Once the resting potential has been restored, removal of excess TCA cycle intermediates may occur through decarboxylation of malate or oxaloacetate.

Exchange, Cataplerosis, and Pyruvate Recycling

Malic enzyme and PEPCK are reversible enzymes. Therefore, isotopically labeled CO_2 may exchange with the unlabeled 4th carbon (a carboxylic group) in malate or oxaloacetate. The result is that malate or oxaloacetate become radiolabeled without a net increase in the pool sizes of these intermediates. Similarly, labeled pyruvate or phosphoenolpyruvate may exchange with the carbons 1–3 in malate and oxaloacetate, respectively. There is experimental evidence that with an excess of pyruvate, the exchange of pyruvate with carbons 1–3 in malate is more rapid than that of CO_2 with the fourth carbon, whereas with an excess of CO_2, the fourth carbon exchanges more rapidly *(92,93).* In vitro the PEPCK exchange reaction greatly exceeds both the carboxylation and the decarboxylation reactions *(29).* Extensive exchange of pyruvate and malate, presumably through neuronal malic enzyme, was inferred from the labeling pattern obtained after intravenous injection of [3-^{13}C]lactate *(2).* It cannot be excluded, however, that carboxylation and decarboxylation in vivo occur through different pyruvate-carboxylating enzymes, such as malic enzyme and PEPCK, or through the mitochondrial and cytosolic isoforms of malic enzyme, so that what looks like exchange is really some cyclic or shuttle-like reaction. For instance, carboxylation in the cytosol and decarboxylation in the mitochondria through cytosolic and mitochondrial malic enzyme, respectively, would produce intramitochondrial NADPH from cytosolic NADPH. Further, if at the same time, some cells are carboxylating pyruvate during activation and others are decarboxylating surplus malate after a resting state has been achieved, this may appear as exchange when whole tissue is analyzed.

Decarboxylation of malate or oxaloacetate is cataplerosis, the opposite of anaplerosis, and it has been observed in neural tissue both in vitro and in vivo. When brain slices are exposed to isotopically labeled GABA, glutamate, citrate, or acetate, labeled lactate or alanine are formed *(36,64).* Similarly, when cultured astrocytes are exposed to ^{13}C-labeled glutamate or acetate ^{13}C- labeled lactate is produced *(13,82,83).* These reactions all involve formation of pyruvate from a 4-carbon intermediate of the TCA cycle (malate or oxaloacetate) by decarboxylation. In vivo it was found that ^{13}C-labeled acetate was converted into lactate *(94),* and that [3-^{13}C]lactate was rapidly metabolized to [2-^{13}C]lactate *(2);* both findings indicated formation of pyruvate by decarboxylation of malate.

Decarboxylation of malate to pyruvate and metabolism of this pyruvate through pyruvate dehydrogenase, so-called pyruvate recycling, was inferred from NMR data obtained in vivo *(4,95).* The authors used the glia-specific substrate acetate, which was doubly ^{13}C-labeled, [1,2-^{13}C]acetate (*see* Fig. 3). This led, as

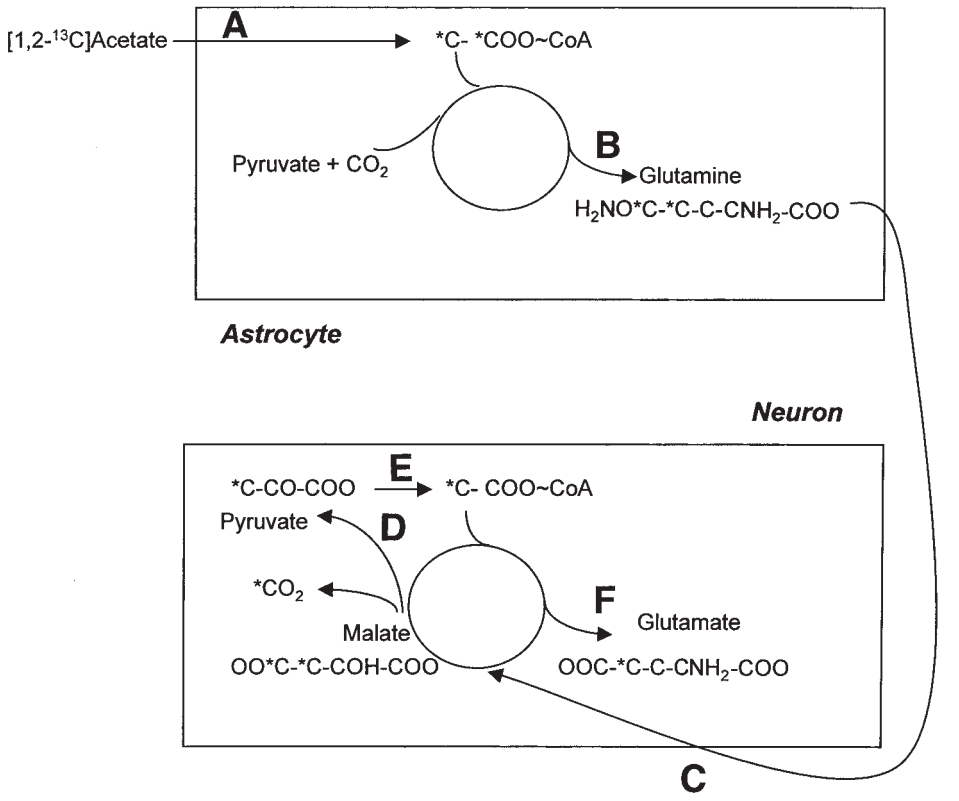

Fig. 3. Carboxylation and decarboxylation in different cell types. Doubly labeled acetate enters astrocytes **(A)** and is converted into doubly labeled glutamine **(B),** which is transferred to neurons **(C),** where it is metabolized through the neuronal TCA cycle to malate. Malate is decarboxylated to singly labeled pyruvate **(D),** which is converted to acetyl~CoA **(E)** and singly labeled glutamate. Based on the data published in refs. *(83)* and *(95).*

expected, to doubly labeled glutamine and glutamate, [4,5-^{13}C]glutamate and [4,5-^{13}C]glutamine, and, more surprisingly, to singly labeled glutamate [4-^{13}C]glutamate, but not to singly labeled glutamine, [4-^{13}C]glutamine. Such labeling may be interpreted in two slightly different ways: [1,2-^{13}C]Acetate is taken up into glia, metabolized through the TCA cycle, and converted to [4,5-^{13}C]glutamate and [4,5-^{13}C]glutamine; the latter is transferred to neurons, fed into the neuronal TCA cycle to become malate, decarboxylated to pyruvate, half of which will be [3-^{13}C]pyruvate, which is metabolized through pyruvate dehydrogenase to [2-^{13}C]acetyl~CoA, which gives [4-^{13}C]glutamate. Alternatively, [1,2-^{13}C]acetate is metabolized to malate through the glial TCA cycle, malate is decarboxylated in glia and gives [3-^{13}C]pyruvate and hence [3-^{13}C]lactate, which is transferred to neurons. In both cases, the loss of malate must be compensated through glial anaplerosis, possibly through pyruvate carboxylase activity. In the first case, neurons will obtain NADPH and pyruvate at the expense of glial ATP (pyruvate carboxylase consumes ATP). In the second case, glial cells spend ATP and obtain NADPH, and if lactate is exported, neurons obtain glial reducing equivalents when lactate is taken up and converted to pyruvate and NADH. Pyruvate recycling in neurons may be inferred from the study of O'Neal and Koeppe *(96)* who gave [2-^{14}C]lac-

tate to rats and recovered some of the label in the 4th carbon of glutamate, and it has been shown in vitro in rat brain mitochondria and in cultured astrocytes *(97,98).*

As pointed out in the Introduction, fixation of CO_2 in the brain must be balanced by complementary decarboxylation reactions, because the cerebral formation of CO_2 is equal to the consumption of O_2. The above reactions illustrate how carboxylation and decarboxylation may occur in different cell types and serve different cellular purposes.

Three Sources of Error in Isotopic Labeling Studies of Anaplerotic Carboxylation

Some interesting sources of error may come into play when isotopic labeling is used to investigate the cellular compartmentation of pyruvate carboxylation in the brain. The first is related to the interpretation of radiolabeling of glutamate and glutamine. Some energy substrates are metabolized predominantly by glia (e.g., acetate), or neurons (e.g., lactate), whereas others, such as glucose, are metabolized by both cell types *(2,66,75,96,99).* When a radiolabeled glial energy substrate is administered to the brain, the specific activity of glutamine becomes higher than that of (whole brain) glutamate. This is so, because only the small glial pool of glutamate becomes labeled and hence glutamine which is formed exclusively in glia *(80,81).* But the large neuronal pool of glutamate remains unlabeled (until labeled glutamine is transferred to neurons and is deamidated to "neuronal" glutmate), and when amino acid labeling in whole brain is analyzed, the large, unlabeled neuronal pool of glutamate will dilute the small, labeled glial pool, and the specific activity of glutamate will become lower than that of the undiluted glutamine. In the classical literature on metabolic compartmentation in the brain, glial metabolism of a radiolabeled substrate is shown by the glutamine/glutamate relative specific activity (glutamine RSA) being higher than 1. Administration of radiolabeled bicarbonate to brain tissue may give a glutamine RSA greater than one *(58);* this has been taken to reflect the purely glial nature of pyruvate carboxylation. It should be obvious, however, that as long as glial pyruvate carboxylation exceeds any neuronal pyruvate carboxylation (expressed as pyruvate carboxylation/TCA cycle rate), the glutamine RSA will remain greater than 1. For instance, if neuronal pyruvate carboxylation is half that of glial pyruvate carboxylation (relative to the TCA cycle rate), the glutamine RSA may be ~2 with $H^{14}CO_3^-$ as the labeled substrate.

A second source of error is related to the metabolic fate of the radiolabeled CO_2 or bicarbonate once it has become a carboxylic group. Through carboxylation $H^{14}CO_3^-$ becomes the fourth carbon of malate or oxaloacetate (Fig. 2). Eventually this carbon will become the first carbon of glutamate and glutamine. But malate (and oxaloacetate after conversion to malate) may equilibrate with fumarate through the freely reversible fumarase reaction (Fig. 2). Fumarate is symmetrical, and the label will therefore become randomized between the first and the fourth positions of fumarate, malate and oxaloacetate. The first carbon is lost as CO_2 in the isocitrate dehydrogenase reaction. Therefore, if equilibration of malate with fumarate is more extensive in one cell type than in another, then radiolabeling from $H^{14}CO_3^-$ will favor detection of pyruvate carboxylation in the latter. It has been shown in vitro that equilibration between malate and fumarate is almost complete in neurons and less extensive in astrocytes *(100).* Equilibration of malate with fumarate in neurons in vivo was shown by radiolabeling of glutamate after intracerebral injection of pyruvate labeled in the carboxyl group *(1).* When carboxylated, this pyruvate leads to formation of malate labeled in the first carbon, the carbon that is lost as CO_2 in the isocitrate dehydrogenase reaction. For [1-^{14}C]pyruvate to label glutamate and glutamine, the malate *must* equilibrate with fumarate so that some label is

transferred to the fourth carbon in malate and hence to the first carbon of glutamate. The several NMR-based calculations of glial pyruvate carboxylase activity in vivo *(66,101–103)* all assume that equilibration with fumarate does not take place in astrocytes. In some of these studies, ^{13}C NMR spectroscopy was performed after administration of [1-^{13}C]glucose, and the stronger labeling of the second than of the third carbon of glutamine was used to calculate the magnitude of glial pyruvate carboxylation (see refs. *66* or *102* for details). Similarly, the near-equal labeling of the second and third carbon positions in glutamate and the (corresponding) third and fourth positions in GABA was taken to confirm the absence of pyruvate-carboxylating activity in neurons. Recently, Bouzier et al. *(99)* made the same assumption when they maintained that lactate is metabolized in a brain compartment that does not have pyruvate carboxylase activity, i.e., neurons. Although the statement is correct in a narrow sense, viz, that neurons do not express pyruvate carboxylase, it is not correct that the neuronal metabolism of lactate does not involve pyruvate carboxylation. This was shown in awake mice *(2)* and in anesthetized rats *(1).*

A third source of error is related to cell-specific isotopically labeled molecules. O'Neal and Koeppe *(96)* gave [2-^{14}C]lactate to rats and their results were interpreted as showing that at least 10% of the lactate was metabolized through pyruvate carboxylation *(91).* Lactate is almost exclusively metabolized by neurons *(2,99);* therefore, neuronal pyruvate carboxylation was probably detected by this method. If one is to estimate carboxylation of propionyl~CoA from the metabolism of labeled propionate, one must keep in mind that short-chain fatty acids are, like acetate, metabolized primarily or exclusively by glia, possibly due to differences in monocarboxylate transport in neurons and glia *(104).* Anesthetics may influence carboxylation in a cell-specific manner. Cheng et al. *(105)* found that thiopental led to a preferential increase in the incorporation of $H^{14}CO_3^-$ into glutamine. Barbiturate anesthesia reduces cerebral glucose metabolism by 50% *(101),* presumably because neurotransmission is reduced. Anesthesia may therefore selectively reduce the neuronal carboxylation that serves formation of transmitter glutamate and GABA. This should be kept in mind when pyruvate carboxylation is assessed in anesthetized animals *(99,102).*

An Attempt at Quantifying Neuronal Pyruvate Carboxylation

In the following section, we will use the available data to calculate the flux of pyruvate through the carboxylation pathways, keeping in mind that there are many reasons for caution. With NMR spectroscopy, glial pyruvate carboxylation has been estimated at 25–40% of the glial TCA cycle activity; in these studies one has assumed that equilibration of oxaloacetate with fumarate does not occur in glia *(101–103).* Equilibration does occur, however, and label randomization increases with the duration of the experiment *(106).* Using the glial randomization value of 40% reached by Merle et al. *(100)* in vitro, the actual glial pyruvate-carboxylating activity could be 42–67% of the glial TCA cycle (as determined from the published values of 25–40%). In a short-term experiment (5 min) Hassel et al. *(66)* arrived at a value for glial pyruvate carboxylation of ~60% of the glial TCA cycle rate, which agreed with the calculated efflux of TCA cycle intermediates from the glial TCA cycle in awake mice. Let us assume a glial pyruvate-carboxylating activity that is 60% of the glial TCA cycle rate, what is then the neuronal pyruvate-carboxylating activity? Waelsch et al. *(58)* found that the glutamine RSA after infusion of $NaH^{14}CO_3^-$ was 1.2–1.9, meaning that the specific activity of glutamate from $NaH^{14}CO_3^-$ was at least 50% of that of glutamine. We have to correct for the glial pool of glutamate (which may be as high as 20% of the total brain pool of glutamate, and

with approximately the same specific activity as glutamine [*58*]*). This leaves us with a specific activity of the remaining, presumably neuronal, pool of glutamate of 40% of that of glutamine. We may assume that the [^{14}C]malate formed from NaH$^{14}CO_3^-$ in neurons underwent complete equilibration with fumarate *(2,99),* meaning that half the label was lost in the isocitrate dehydrogenase reaction. This translates into a neuronal pyruvate carboxylating activity that is (60% × 40% × 2=) 48% of the neuronal TCA cycle rate. This value agrees reasonably well with NMR data obtained in awake mice after short-term exposure to the neuronal substrate [3-^{13}C]lactate *(2):* when the neuronal TCA cycle activity was blocked at the succinate dehydrogenase step with 3-nitropropionic acid, pyruvate carboxylation maintained label incorporation at 30–40% of control. It has been calculated that glutamine could account for only two-thirds of the GABA formation in awake mice *(107);* the remaining one-third could therefore be explained by neuronal pyruvate carboxylation. Such a high anaplerotic activity is within the limits of the carboxylating activity of malic enzyme: whole-tissue malic enzyme activity (carboxylating) was 60% of α-ketoglurate dehydrogenase activity in rat striatum *(1).* In vitro the magnitude of pyruvate carboxylation was estimated at one-third of the TCA cycle activity in cultured cerebellar granule neurons. The calculation was based on the labeling of aspartate from [1-^{14}C]pyruvate and [2-^{14}C]pyruvate; the latter label enters the TCA cycle both through pyruvate dehydrogenase and through carboxylation.

These values for cerebral pyruvate carboxylation are much higher than the value of 10%, which is frequently encountered in the literature, often with a reference to Berl et al. *(106)* or Waelsch et al. *(58),* although none of these papers calculate anaplerotic activity relative to metabolism of glucose.

The cerebral TCA cycle rate has been determined from the ^{13}C labeling of glutamate from [1-^{13}C]glucose *(108,109).* [1-^{13}C]Glucose yields [3-^{13}C]pyruvate and hence [2-^{13}C]acetyl~CoA, and it rapidly labels glutamate in the fourth carbon position. Only after a while do the second and third positions in glutamate become labeled, a temporal pattern that has been interpreted to reflect the cycling of label in the TCA cycle *(66,101,102,110).* But the second and third positions of glutamate would also become labeled if the [3-^{13}C]pyruvate were carboxylated to malate with subsequent equilibration with fumarate. The delay in the labeling of glutamate in the second and third positions could well be due to the time needed for malate to equilibrate with fumarate + for oxaloacetate to equilibrate with aspartate. From a temporal point of view the difference between the straightforward operation of the TCA cycle and the pyruvate carboxylation pathway is the time needed for label to equilibrate with the pools of glutamate and succinate (Fig. 1). Does the high pyruvate-carboxylating activity calculated above affect previous calculations of the TCA-cycle rate? Not really, because the labeling of glutamate in the second and third positions, be it through pyruvate carboxylation or cycling of label, still reflects the flux through citrate synthase, aconitase, and isocitrate dehydrogenase.

Some factors make the quantitation of neuronal pyruvate carboxylation uncertain. First, the calculation is based on a glutamine RSA of 1.9 as reported by Waelsch et al. *(58),* but they also reported lower values in several experiments, meaning that the neuronal carboxylating rate could be higher than that calculated above. Second, the calculation is based on val-

*The glutamate pool in glia may be estimated from the labeling of glutamine and glutamate by a strictly glial substrate. Three times as much label arrives in glutamine than in glutamate when one administers isotopically labeled acetate *(94)* or radiolabeled bicarbonate together with ammonia to stimulate glial carboxylation *(58).* If glial glutamate, therefore, is one-third of the brain glutamine pool, which itself is ~60% as large as the total glutamate pool, then glial glutamate accounts for approx 20% of whole brain glutamate. When glutamine is radiolabeled three times as strongly as glutamate and has a pool size that is 60% of that of whole-brain glutamate, then the glutamine RSA becomes 5.

ues obtained in cat cerebral cortex and in whole mouse brain, and these values may not be representative for all brain regions. For instance, radiolabeled bicarbonate gives a glutamine RSA < 1 when injected into striatum *(1,2),* meaning that neuronal carboxylation predominates over glial carboxylation in this region. Further white matter, which constitutes 50% of the human brain *(111),* and which has an oxidative activity that is ~20% of gray matter *(112,113),* may have a much lower pyruvate-carboxylating activity than the cerebral cortex. Third, exchange reactions, which may be difficult to quantify, may lead to overestimation of anaplerotic activity.

The Amino Group

Until now we have considered only the carbon skeleton of α-ketoglutarate and its derivatives, disregarding the amino group of glutamate and GABA. Neuronal formation of glutamate from α-ketoglutarate may occur through transamination or reductive amination. Transamination requires neuronal uptake of amino donors, e.g., branched-chain amino acids that could originate from the circulation *(9),* or alanine that could be released from glia or from other neurons *(115).* Reductive amination, which is the formation of glutamate from α-ketoglutarate and NH_3 by glutamate dehydrogenase, does not require amino donors. The relative importance of transaminastion and reductive amination for the formation of transmitter glutamate and GABA is not known; both alanine aminotransferase *(116),* branched-chain amino acid transferase *(117),* and glutamate dehydrogenase *(118)* have been demonstrated in nerve terminals.

Summary

Anaplerosis in the brain includes carboxylation of pyruvate, propionyl~CoA and possibly phosphoenolpyruvate, in addition to pathways that do not involve carboxylation. Anaplerotic carboxylation reactions are probably balanced by complementary cataplerotic decarboxylation reactions, because the cerebral formation of CO_2 equals the consumption of O_2. Carboxylation and decarboxylation may lead to exchange reactions, but may also occur in separate cells, or cellular compartments through different enzymes. The two reactions may also represent different activational states of the cell, because anaplerotic carboxylation would increase the oxidative capacity of the cell during increased energy requirement, and because decarboxylation may remove surplus TCA cycle intermediates in the resting state of the cell. Neurons and glia differ with respect to equilibration of malate and oxaloacetate with fumarate. This may affect the labeling efficacy of the carboxylation substrates $H^{14}CO_3^-$ and [1-^{14}C]pyruvate, so that detection of glial pyruvate carboxylation is favored with the use of $H^{14}CO_3^-$ and detection of neuronal pyruvate carboxylation is favored with the use of [1-^{14}C]pyruvate. Neuronal carboxylation of pyruvate has been shown in vivo and in vitro; it may account for approximately 30% of the neuronal TCA cycle rate, at least in some populations of neurons, whereas glial pyruvate carboxylation may amount to 40–60% of the glial TCA cycle rate. Anaplerotic carboxylation in neurons has a role in compensating for the loss of α-ketoglutarate inherent in glutamatergic and GABAergic neurotransmission, and it explains the apparent lack of glutaminase in some populations of glutamatergic and GABAergic neurons.

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