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Metabolic Regulation of Neocortical Expansion in Development and Evolution

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Summary

The neocortex, the seat of our higher cognitive abilities, has expanded in size during the evolution of certain mammals such as primates, including human. This expansion occurs during development and is linked to the proliferative capacity of neural stem and progenitor cells (NPCs) in the neocortex. A number of cell-intrinsic and cell-extrinsic factors have been implicated in increasing NPC proliferative capacity. However, NPC metabolism has only recently emerged as major regulator of NPC proliferation. In this *Perspective*, we summarize recent insights into the role of NPC metabolism in neocortical development and neurodevelopmental disorders, and its relevance for neocortex evolution. We discuss certain human-specific genes and microcephaly-implicated genes that operate in, or at, the mitochondria of NPCs and stimulate their proliferation by promoting glutaminolysis. We also discuss other metabolic pathways and develop a perspective how metabolism mechanistically regulates NPC proliferation in neocortical development, and how this contributed to neocortex evolution.

Introduction

The neocortex is the brain region that underwent major changes in size and morphology during primate evolution (Rakic, 2009) (**Fig. 1**). Compared to other primates, human has the largest neocortex with a highly convoluted morphology, referred to as gyrification (Cardenas and Borrell, 2020; LaMonica et al., 2012; Namba and Huttner, 2017; Rash et al., 2019; Sun and Hevner, 2014). Since the neocortex is the seat of our higher cognitive functions, understanding human neocortex development and evolution will provide insight into what makes us human.

Neocortical expansion in human is associated with extensive neuron production, which in turn reflects an increase in the number of neural stem and progenitor cells (NPCs) during development (**Fig. 1**). In the developing neocortex, there are two major classes of NPCs, referred to as apical progenitors (APs) and basal progenitors (BPs) (LaMonica et al., 2012; Namba and Huttner, 2017). APs are located in the apical-most germinal zone, the ventricular zone (VZ), and undergo mitosis at the ventricular (apical) surface. With the onset of neurogenesis, APs comprise two major types, apical radial glia (aRG, also known as ventricular radial glia) and apical intermediate progenitors (LaMonica et al., 2012; Namba and Huttner, 2017). At the early stage of neocortical development, aRG expand their number by symmetric proliferative divisions and then give rise to secondary progenitors, that is, the BPs. BPs undergo mitosis in the subventricular zone (SVZ), which is located basally to the VZ (Bystron et al., 2008). In species with a large neocortex, the SVZ is further subdivided into an inner and an outer SVZ (ISVZ and OSVZ, respectively), which reflects the expansion of BPs (Smart et al., 2002). There are two major types of BPs, basal intermediate progenitors (bIPs) and basal radial glia (bRG, also known as outer radial glia) (LaMonica et al., 2012; Namba and Huttner, 2017).

In mouse embryonic neocortex, the majority of bIPs are neurogenic, i.e. divide once giving rise to two post-mitotic neurons, whereas in human fetal neocortex, bIPs exhibit proliferative capacity. Furthermore, human fetal neocortex shows an abundance of bRG, which exhibit high proliferative capacity. Since mammals with a relatively small neocortex, such as mouse, in general have very few bRG and proliferative bIPs (Shitamukai et al., 2011; Vaid et al., 2018; Wang et al., 2011), the evolutionary enlargement of the neocortex can be attributed to an expansion of proliferative bIPs and bRG (LaMonica et al., 2012; Namba and Huttner, 2017).

How BPs expand in number during human neocortical development has been a long-standing question. There are basically two approaches that have been taken to address it. One has been studying the etiologies of human neocortical malformations such as microcephaly (Juric-Sekhar and Hevner, 2019; Pinson et al., 2019; Verloes et al., 2013). At least 18 genes implicated in primary (congenital) microcephaly (MCPH) when mutated, that is, genes responsible for neocortex growth during development, have been identified (Jayaraman et al., 2018). The other approach has been exploring the role of human-specific genes that are preferentially expressed in NPCs of human fetal neocortex. Four recent papers identified two human-specific genes involved in BP abundance and neocortical expansion, *ARHGAP11B* (Florio et al., 2015) and *NOTCH2NL* (Fiddes et al., 2018; Florio et al., 2018; Suzuki et al., 2018). Of note, among the MCPH genes and human-specific genes, *MCPH1* and *ARHGAP11B*, respectively, have very recently been implicated in mitochondrial function and NPC metabolism (Journiac et al., 2020; Namba et al., 2020). These findings prompt us to review (i) the role of NPC metabolism in neocortical development and (ii) the significance of

aberrant NPC metabolism for neurodevelopmental disorders, and to present a perspective on the metabolic regulation of human neocortical evolution.

NPC Metabolism in Physiological and Pathological Neocortical Development – Insights and Perspectives

NPC metabolism in neocortical development has been studied mainly in mouse. Previous studies have suggested that the different types of NPCs, studied both during development and in the adult, exhibit distinct cell metabolism (Beckervordersandforth et al., 2017; Khacho et al., 2016; Llorens-Bobadilla et al., 2015; Shin et al., 2015). It is therefore constructive for this *Perspective* to describe the different metabolic pathways that have been studied in NPCs, and to outline their relevance for neocortical development. To provide an overview, **Table 1** summarizes the activities of each metabolic pathway in mouse and human NPCs. In addition, for each metabolic pathway, we shall also discuss the significance of its aberration for neurodevelopmental disorders.

Glycolysis, Oxydative Phosphorylation, and the Role of Disruption of the TCA Cycle in Microcephaly

Glucose is a main source of energy in metabolism. Glucose taken up by cells is used for glycolysis, a catabolic pathway that produces ATP and pyruvate or lactate, the latter being produced in anaerobic conditions (**Fig. 2**). Pyruvate is transported into mitochondria and then converted into acetyl-CoA (intramitochondrial production). Acetyl-CoA fuels the tricarboxylic acid (TCA) cycle to produce NADH. NADH is then used for oxidative phosphorylation (OXPHOS), which consumes O_2 and NADH to produce ATP. In contrast, under anaerobic conditions, pyruvate is preferentially converted into lactate. However, this preferential lactate production from glucose via pyruvate can also happen under aerobic conditions. This is also known as the Warburg effect, and notably occurs in rapidly dividing

progenitor cells and cancer cells (DeBerardinis and Chandel, 2020; Liberti and Locasale, 2016).

The Balance between Glycolysis and Oxydative Phosphorylation.

A pioneering paper by Ruth Slack and colleagues described that in the mouse embryonic neocortex, the metabolism between self-renewing NPCs (aRG) and differentiated NPCs (bIPs) is different (Khacho et al., 2016). aRG show higher glycolysis with elevated lactate production and lower OXPHOS. In contrast, bIPs exhibit lower glycolysis and higher OXPHOS. Because angiogenesis in the neocortex only increases at mid-neurogenesis (Komabayashi-Suzuki et al., 2019; Lange et al., 2016), the expansion of aRG during the early phase of neocortical neurogenesis presumably occurs under a hypoxic condition. Evidence in support of this notion is the induction of HIF-1α expression in aRG (Komabayashi-Suzuki et al., 2019; Lange et al., 2016) and the accumulation of glycogen in aRG (Gressens and Evrard, 1993). In contrast, the level of bIPs only increases at later stages of cortical neurogenesis. Furthermore, after the onset of angiogenesis, the density of blood vessels in the SVZ is greater than in the VZ (Komabayashi-Suzuki et al., 2019; Lange et al., 2016). This, too, would favour a higher OXPHOS in bIPs than aRG.

Consistent with these findings, the manipulation of the glycolysis vs. OXPHOS balance in mouse embryonic neocortex has been shown to affect NPC proliferation vs. differentiation (Khacho et al., 2017). This manipulation involved the Apoptosis Inducing Factor (AIF), a mitochondrial protein that is essential for the activity of complex I of the respiratory chain. Disruption of the *AIF* gene was found to promote glycolysis and NPC proliferation and to impair the generation of neurons from the NPCs (Khacho et al., 2017). As a consequence of this decrease in neuron production, the *AIF* KO mice have a thinner neocortex.

Similar differences in metabolism between self-renewing NPCs and their more differentiated progeny have also been reported for various neuronal systems in other species, including *Drosophila* (Agathocleous et al., 2012; Homem et al., 2014; Zheng et al., 2016). Thus, in general, self-renewing NPCs exhibit higher glycolysis and lower OXPHOS than more differentiated NPCs or neurons. Interestingly, when a high rate of glycolysis ending at lactate is artificially sustained in human iPSC-derived NPCs upon induction of neuronal differentiation, this impairs proper differentiation and results in cell death. Hence, the metabolic shift from glycolysis to OXPHOS is important for neuron production (Zheng et al., 2016). Similarly, *Drosophila* NPCs have high glycolysis, and inhibition of the TCA cycle by knock-down of the alpha-ketoglutarate-dehydrogenase-complex (see **Fig. 2**) reduces the neurogenesis from these NPCs. This, again, suggests that the metabolic shift from glycolysis to OXPHOS is critical for the switch from NPC proliferation to neuron production (Homem et al., 2014). Taken together, these studies suggest that the balance of glycolysis vs. OXPHOS, which presumably is regulated by oxygen availability in the germinal zones, is a crucial regulator of NPC proliferation vs. differentiation.

Role of Aldehyde Dehydrogenase in the Control of the Glycolysis vs. Oxydative Phosphorylation Balance.

The balance of glycolysis vs. OXPHOS is affected by the level of various metabolites. One is related to the activity of aldehyde dehydrogenase Aldh1a3, an enzyme involved in multiple metabolic pathways (Mao et al., 2013). Aldh1a3 increases the concentration of acetate, which then is converted to acetyl-CoA. Since Aldh1a3 provides additional acetyl-CoA, the other major source of acetyl-CoA, that is, glycolysis-derived pyruvate, can be used for lactate production. In other words, glycolysis ending at lactate is enhanced by the activity of Aldh1a3

(Mao et al., 2013). This activity is indirectly affected by Sam68, an RNA binding protein. Specifically, Sam68 regulates the splicing of *Aldh1a3* mRNA, as follows. In mouse embryonic neocortex, Sam68 expression is high and thereby ensures the expression of full-length *Aldh1a3* mRNA in NPCs (La Rosa et al., 2016). In contrast, in Sam68-deficient mouse neocortex, Aldh1a3 is expressed as a C-terminally truncated isoform instead of a full-length protein. This truncated isoform has lower activity, and thus glycolysis ending at lactate is reduced. As a result, the NPCs no longer proliferate but generate neurons (La Rosa et al., 2016). This study therefore provides further evidence for a role of glycolysis ending at lactate in neocortical NPC proliferation.

How is Glycolysis Ending at Lactate Linked to NPC Proliferation?

In light of these data, the crucial question arises: why is glycolysis ending at lactate beneficial for NPC proliferation? As is also addressed below in the section *Glutaminolysis and its Roles in Neocortical Development and its Malformations*, an increase in lactate production would reduce the level of pyruvate and consequently of acetyl-CoA, and hence the occurrence of the first step of the TCA cycle, the formation of citric acid from oxaloacetate (**Fig. 2**). By then fueling the TCA cycle via glutaminolysis (see below), the TCA cycle would be converted from a full cycle to a "three-quarter cycle" metabolic pathway that starts at the α-ketoglutarate (α-KG) step and stops at the oxaloacetate step, with the oxaloacetate now being available for reactions other than citric acid formation (**Fig. 3**). Importantly, the oxaloacetate could now be used as a primary metabolic source for anabolic processes such as the synthesis of nucleotides and various amino acids (**Fig. 3**). In this way, a "three-quarter TCA cycle" metabolic pathway would promote cell cycle progression by supporting these anabolic processes.

MITOCHONDRIAL THIAMINE PYROPHOSPHATE CARRIER SLC25A19 AND AMISH LETHAL MICROCEPHALY.

Primary (congenital) microcephaly is known to be caused by impairment in NPC proliferation (Duerinckx and Abramowicz, 2018; Juric-Sekhar and Hevner, 2019; Pinson et al., 2019). Most genes implicated in microcephaly encode proteins associated with the centrosome, providing a link to NPC division. However, there are two proteins encoded by genes implicated in microcephaly that have been shown to be involved in mitochondrial function and metabolism, MCPH1 (please see below) and SLC25A19 (to be discussed now).

A major form of microcephaly that is associated with a mitochondrial function deficiency is the Amish lethal microcephaly caused by mutation of the *SLC25A19* gene, which results in reduced SLC25A19 protein expression (Bottega et al., 2019; Kelley et al., 2002; Rosenberg et al., 2002). SLC25A19 is a mitochondrial thiamine pyrophosphate carrier. Thiamine pyrophosphate (TPP) is a coenzyme of the α-ketoglutarate dehydrogenase (KGDH) complex that operates in the TCA cycle (**Fig. 2**). Hence, the reduction in SLC25A19 expression results in decreased KGDH activity. As a decrease in KGDH activity leads to microcephaly (Yoon et al., 2017), this raises the possibility that NPC proliferation may not only be promoted by glycolysis ending at lactate, as discussed above, but may also require αKG catabolism. This possibility would be consistent with the findings that glutaminolysis, which provides αKG, is important for NPC proliferation in the developing neocortex, including human (Journiac et al., 2020; Namba et al., 2020).

Glutaminolysis and its Roles in Neocortical Development and its Malformations

Glutaminolysis is a metabolic pathway that converts glutamine to glutamate to α-KG (**Fig. 2**). Glutaminolysis is an important pathway to fuel the TCA cycle independent of glycolysis. Glutaminolysis is a hallmark of cells with high proliferative capacity, notably cancer cells and embryonic stem cells (Yang et al., 2017). Thus, two metabolic pathways are characteristically observed in highly proliferating cells, glycolysis ending at lactate, and glutaminolysis.

As already mentioned above, the combination of these two metabolic pathways in highly proliferating cells likely promotes cell cycle progression by supporting a variety of anabolic processes that are required for cell replication. Interestingly, two independent recent studies have shown that glutaminolysis plays an important role in NPC proliferation in the developing neocortex. One study (Journiac et al., 2020) showed that a protein encoded by a gene implicated in primary microcephaly, Mcph1, in mouse embryonic neocortex is located on mitochondria and stimulates aRG proliferation via glutaminolysis. The other study (Namba et al., 2020) showed that a protein encoded by a human-specific gene implicated in the evolutionary expansion of the human neocortex, ARHGAP11B, is localized in mitochondria and stimulates BP proliferation via glutaminolysis. Thus, two genes implicated in the determination of brain size operate by promoting the proliferation of the two principal classes of NPCs via stimulation of the metabolic pathway of glutaminolysis.

Here we summarize the molecular functions of ARHGAP11B (Namba et al., 2020) and Mcph1 (Journiac et al., 2020) in mitochondrial metabolism. As is described in detail below, both proteins are associated with mitochondria and increase the mitochondrial calcium concentration, and we discuss how the increase in mitochondrial calcium concentration likely promotes glutaminolysis.

ARHGAP11B ACTION IN MITOCHONDRIA.

ARHGAP11B is a human-specific gene that arose around 5 million years ago, after the split of the lineages leading to modern human and chimpanzee (Antonacci et al., 2014; Prüfer et al., 2014; Sudmant et al., 2010). *ARHGAP11B* originated by a partial gene duplication from the widespread gene *ARHGAP11A*, which encodes a Rho family small GTPase activating protein (RhoGAP) (Kagawa et al., 2013; Zanin et al., 2013) that is localized in the nucleus (Namba et al., 2020). Like the ARHGAP11A protein, the ancestral ARHGAP11B protein (that is, as it arose originally), which contains the full RhoGAP domain of ARHGAP11A, is localized in the nucleus and exhibits RhoGAP activity in vivo, but does not stimulate BP proliferation (Florio et al., 2016; Namba et al., 2020). Subsequent to its genesis, the ancestral *ARHGAP11B* gene was subject to a single base substitution (Florio et al., 2016) that had dramatic consequences. This single base substitution generated a new splice donor site, the use of which results in a protein, referred to as modern ARHGAP11B, that contains a novel, human-specific 47-amino acid C-terminal domain and a truncated RhoGAP domain and that lacks RhoGAP activity in vivo (Florio et al., 2016). Importantly, modern ARHGAP11B is imported into the mitochondrial matrix due to the unmasking of an N-terminal mitochondrial import sequence (Namba et al., 2020), and does stimulate BP proliferation, a hallmark of neocortical expansion, in various model systems(Florio et al., 2015; Heide et al., 2020; Kalebic et al., 2018; Namba et al., 2020).

Inside the mitochondrial matrix, modern ARHGAP11B binds to adenine nucleotide translocators (ANTs) (Namba et al., 2020). ANTs have two distinct functions. One is exchanging ADP and ATP (Halestrap and Richardson, 2015), and the other is the regulation of the mitochondrial permeability transition pore (mPTP) (Doczi et al., 2016; Halestrap and Richardson, 2015). ARHGAP11B does not affect ADP/ATP translocation, but inhibits mPTP

opening. This inhibition is mediated by the human-specific C-terminal domain of modern ARHGAP11B. An opening of the mPTP leads to the release of calcium from mitochondria, and an inhibition of mPTP opening has been shown to result in a higher mitochondrial calcium concentration (Brookes et al., 2004; De Marchi et al., 2014). Consistent with this, mitochondria of ARHGAP11B-expressing cells show an increase in their calcium concentration (Namba et al., 2020).

ARHGAP11B-expressing cells show enhanced glutaminolysis (Namba et al., 2020). Inhibition of glutaminolysis in human fetal neocortex causes a reduction in the abundance of proliferating BPs. Consistent with this, the increase in BP mitoses upon ARHGAP11B expression in mouse embryonic neocortex is completely abolished by inhibition of glutaminolysis. These data therefore show that glutaminolysis is a major metabolic pathway promoting BP proliferation in human developing neocortex (Namba et al., 2020).

The mechanism how ARHGAP11B promotes glutaminolysis, and the link between the increased mitochondrial calcium concentration and stimulation of glutaminolysis by ARHGAP11B (Namba et al., 2020), are not yet fully understood. Calcium is a known regulator of mitochondrial metabolism (Cannino et al., 2018; Lawlis and Roche, 1981; Rutter and Denton, 1989). Specifically, there are several mitochondrial enzymes which can be activated by calcium (Cannino et al., 2018; Lawlis and Roche, 1981; Rutter and Denton, 1989). One of these enzymes is α-ketoglutarate dehydrogenase (KGDH, **Fig. 2**) (Cannino et al., 2018; Lawlis and Roche, 1981; Rutter and Denton, 1989). Activation of KGDH would lead to a greater consumption of αKG, which in turn could result in a feed-back stimulation of glutaminolysis to replenish αKG levels.

MCPH1 ACTION AT MITOCHONDRIA.

MCPH1 is one of the well-studied genes which, when mutated, can cause autosomal recessive primary microcephaly (MCPH) (Jackson et al., 1998; Pulvers et al., 2015). *MCPH1* has been shown to be expressed in neocortical NPCs, specifically in aRG of mouse and aRG and bRG of human (Journiac et al., 2020). The MCPH1 protein has been shown to serve multiple functions in genome stability, being involved in DNA damage-induced S and G2/M checkpoints and in chromosome segregation (Liang et al., 2010; Peng et al., 2009; Pulvers et al., 2015). Besides these roles, a recent study has shown that NPCs derived from the embryonic neocortex of a Mcph1 knockout mouse exhibit fragmented mitochondria and defects in mitochondrial metabolism (Journiac et al., 2020). Surprisingly, in contrast to previous observations (Liang et al., 2010; Peng et al., 2009; Pulvers et al., 2015), Mcph1 was found to be localized in association with mitochondria (Journiac et al., 2020). Of note, Mcph1 interacts with both VDAC1, an ion channel in the mitochondrial outer membrane, and glucose-regulated protein 75 (GRP75) (Journiac et al., 2020), which induces endoplasmic reticulum (ER)-mitochondrial coupling by bridging VDAC1 on mitochondria and the IP3 receptor on the ER (Szabadkai et al., 2006). This ER-mitochondrial coupling allows calcium influx from the ER into mitochondria (Szabadkai et al., 2006). Lack of Mcph1 in the mouse knockout neocortical NPCs results in a reduced mitochondrial calcium level (Journiac et al., 2020), presumably reflecting a reduced calcium influx from the ER into mitochondria. In addition, this lack of Mcph1 (Journiac et al., 2020) is associated with a lack of inhibition of the negative regulator of VDAC1, the AKT-HK2 pathway (Beg et al., 2017; Betz et al., 2013), presumably resulting in reduced activation of VDAC1. In summary, the presence of Mcph1 in neocortical NPCs is required for maintaining a mitochondrial calcium concentration that is required for optimal mitochondrial function (Journiac et al., 2020).

As briefly alluded to above, calcium is a positive regulator of various aspects of mitochondrial metabolism. Mitochondrial calcium levels are also known to be linked to mitochondrial morphology (Golic et al., 2014; Hom et al., 2007). Accordingly, given the decrease in mitochondrial calcium concentration in Mcph1-deficient mouse NPCs, their mitochondrial metabolic activity is impaired and their mitochondria become fragmented (Journiac et al., 2020). These deficiencies eventually lead to decreased NPC proliferation and increased NPC apoptosis, which are well-known causes of primary microcephaly (Duerinckx and Abramowicz, 2018; Juric-Sekhar and Hevner, 2019; Pinson et al., 2019).

In addition to these effects, Mcph1 regulates the expression level of the mitochondrial isoform of phosphoenolpyruvate carboxykinase (PEPCK-M, PCK2) (Journiac et al., 2020), which converts oxaloacetate to phosphoenolpyruvate (Mendez-Lucas et al., 2014). In Mcph1 KO NPCs, the expression of PCK2 is reduced, and there is no upregulation of PCK2 expression upon glucose deprivation, in contrast to what is observed in WT NPCs (Journiac et al., 2020). The expression level of the transcription factor ATF4, which positively regulates PCK2 expression, is also impaired in Mcph1 KO NPCs. These results therefore imply that Mcph1 positively regulates PCK2 expression by increasing ATF4 expression.

Its consumption by PCK2 depletes oxaloacetate from the TCA cycle. Concomitant with an upregulation of PCK2, glutaminolysis is increased (Montal et al., 2015). This in turn will increase the supply of αKG, which is subsequently converted to oxaloacetate, fueling the amino acid and nucleotide synthesis pathways (**Fig. 3**). Given that PCK2 levels are linked to the presence of Mcph1, Mcph1 emerges as a prerequisite for an increase in glutaminolysis, which is crucial for NPC proliferation in the developing neocortex, including human (Journiac et al., 2020; Namba et al., 2020). In this context, it is intriguing to note that the

human MCPH1 protein contains human-specific amino acid changes that were fixed during human evolution (Wang and Su, 2004). It would therefore be interesting to investigate whether these differences are of relevance for mitochondrial metabolism of human NPCs.

Considering the effects of ARHGAP11B and Mcph1 on mitochondrial metabolism together (Journiac et al., 2020; Namba et al., 2020), these studies indicate that glutaminolysis plays an important role in neocortical growth control. Further evidence for an important role of glutaminolysis is provided by a study showing that patients with mutations in the mitochondrial glutamate transporter gene *SLC25A22* exhibit a slightly smaller brain (–2 SD) (Molinari et al., 2009). Glutaminolysis should therefore be taken into account as one of the metabolic pathways whose alterations can impair proper neocortical growth and lead to microcephaly.

Fatty Acid Metabolism and Microcephaly

Fatty acid metabolism comprises fatty acid synthesis and fatty acid catabolism, the latter referred to as β-oxidation (**Fig. 2**). Fatty acid synthesis occurs in the cytoplasm, starting from acetyl-CoA (extramitochondrial production). Conversely, acetyl-CoA production from fatty acids by β-oxidation takes place in mitochondria (intramitochondrial production). Fatty acids are involved in multiple cellular processes, including the lipid modification of various proteins (see below).

Fatty acids, especially those with long acyl chains, need the carnitine shuttle system for their transport from the cytoplasm into mitochondria. One of the essential proteins of the carnitine shuttle system, Cpt1a (**Fig. 2**), is known to be involved in maintaining the aRG pool size in the mouse embryonic neocortex (Knobloch et al., 2017). Thus, knockdown of Cpt1a reduces

the proliferation of aRG. Furthermore, trimethyllysine dioxygenase (TMLHE, **Fig. 2**), the first enzyme in carnitine synthesis, is also crucial for maintaining the aRG pool size in the mouse embryonic neocortex (Bankaitis and Xie, 2019; Xie et al., 2016). Hence, transport of fatty acids along with carnitine into mitochondria, allowing subsequent β-oxidation, plays an important role in the aRG proliferation-differentiation balance.

A mutation in fatty acid synthase (FASN R1819W, **Fig. 2**) is associated with intellectual disability (Najmabadi et al., 2011). Human cerebral organoids expressing FASN R1819W exhibit reduced NPC proliferation (Bowers et al., 2020). In contrast, a transgenic mouse carrying Fasn R1819W shows no differences in aRG and bIP abundance during neocortical development, although this mouse does show a reduction in adult hippocampal neurogenesis (Bowers et al., 2020). These results raise the possibility that fatty acid synthesis may be involved in a specific way in human NPC proliferation.

Palmitoylation, the post-translational modification of proteins by covalent addition of palmitate, has recently been found to be required to maintain the full level of BP proliferation in human fetal neocortex (Kalebic et al., 2019). Pharmacological inhibition of palmitoylation reduced BP abundance in human fetal neocortex tissue *ex vivo*, but not in mouse embryonic neocortex tissue (Kalebic et al., 2019). In line with this finding, human fetal BPs, but not mouse embryonic BPs, express a specific isoform of the morpho-regulatory protein PALMD, which contains a palmitoylation site. Upon palmitoylation, PALMD associates with the plasma membrane, where it increases BP process formation (Kalebic et al., 2019). This increase in the number of cell processes endows the BPs with a greater probability to interact with the extracellular matrix, a positive extracellular cue for BP proliferation. Taking all these findings together, fatty acid metabolism emerges as a very important metabolic pathway in

neocortical neurogenesis and potentially in the evolutionary expansion of the human neocortex, with one underlying mechanism being a modulation of BP morphology.

Defects in fatty acid metabolism have been shown to cause microcephaly One such type of microcephaly is caused by an impairment of fatty acid transport into the developing brain tissue (Guemez-Gamboa et al., 2015). Another such type of microcephaly is caused by deterioration of fatty acid metabolism within the brain. Thus, a patient carrying a deletion of *BBOX1* (butyrobetaine-gamma 2-oxoglutarate dioxygenase 1), a gene encoding an enzyme which catalyzes the last step of carnitine synthesis, has been found to develop microcephaly (Rashidi-Nezhad et al., 2014). These studies suggest that transport of fatty acids along with carnitine into mitochondria, and their subsequent β-oxidation, are crucial for human fetal NPC proliferation.

Cholesterol Metabolism

The importance of cholesterol biosynthesis has been studied in mouse embryonic neocortex. Disruption of cholesterol biosynthesis in mouse neocortical NPCs by a conditional squalene synthase (SQS, **Fig. 2**) knockout results in a smaller neocortex (Saito et al., 2009). This conditional squalene synthase knockout in the NPCs enhances VEGF expression in aRG, which in turn induces aberrant angiogenesis in the VZ. This increased blood vessel formation in the VZ reduces the hypoxic condition of the aRG and thus suppresses the expression of hypoxia-inducible factor (HIF-1) (Komabayashi-Suzuki et al., 2019; Saito et al., 2009). An independent study has shown that HIF-1 is a known positive regulator of glycolysis and that aRG with reduced HIF-1 expression show a lower level of glycolysis and decreased proliferation (Lange et al., 2016). Taken together, these studies provide an explanation why

conditional disruption of cholesterol biosynthesis in mouse neocortical NPCs results in a smaller neocortex.

Cholesterol biosynthesis has also been shown to have a key role in the sonic hedgehog (Shh) signaling pathway (Stottmann et al., 2011). Shh is known to carry covalently bound cholesterol, which is required for its ability to act as a morphogen (Wendler et al., 2006). Consistent with this, in mice lacking Hsd17b7, the enzyme catalyzing the last step of cholesterol biosynthesis, the Shh signaling pathway is impaired (Driver et al., 2016; Stottmann et al., 2011). In the neocortex of these mutant mice, the abundance of aRG is decreased whereas that of BPs is increased. Interestingly, the morphology of the aRG is impaired and shows a disruption of the apical adherens junction belt. This likely results in increased aRG delamination, which would explain the decreased aRG and increased BP abundance.

One Carbon Metabolism, notably Folate Metabolism, and Spina Bifida

One carbon (1C) metabolism comprises the methionine cycle and the folate cycle, and is important for the biosynthesis of purines and thymidine, for amino acid metabolism and for DNA and protein methylation (**Fig. 2**) (Yang and Vousden, 2016). Folate is crucial for neural tube closure, and insufficient folate supply from the mother to the human fetus causes neural tube closure deficiencies such as spina bifida (Greene et al., 2011). It has been shown that the intake of folate before conception and during the first trimester of pregnancy can significantly reduce the risk of spina bifida (Czeizel, 2000; Wilson et al., 2003). In mouse, a knockout of 5,10-methylene tetrahydrofolate reductase (MTHFR), a critical enzyme in the folate cycle (**Fig. 2**), causes a defect in neural tube closure (Leung et al., 2017), corroborating the embryonic folate deprivation hypothesis. This study further showed that glycine metabolism

is crucial for neural tube closure. Thus, a proper 1C metabolism, including the folate cycle, is key for preventing spina bifida. Since 1C metabolism is known to be involved in DNA and protein methylation (see below), there may be epigenetic regulation of neural tube closure (Greene et al., 2011).

A recent study has shown that the abundance of BPs (both bIPs and bRG) is significantly reduced in the neocortex of fetuses afflicted with open spina bifida (Fietz et al., 2020). This report therefore implies that a proper 1C metabolism is also required for the full level of BP proliferation in developing human neocortex. Hence, the folate cycle may be important not only for neural tube closure but also for neocortical expansion, which is thought to involve increased BP proliferation.

The activity of dihydrofolate reductase (DHFR), a key enzyme in 1C metabolism (**Fig. 2**), is controlled by Eph receptor signaling (Fawal et al., 2018). Eph B receptor forward signal in aRG inhibits DHFR expression, which in turn impairs 1C metabolism and eventually decreases H3K4 tri-methylation (H3K4me3). Since H3K4me3 is linked to transcriptional activation, decreased H3K4me3 in aRG may suppress the transcription of genes that are important for NPC proliferation. Indeed, it has been suggested that the promoter activity of several genes expressed in aRG may depend on H3K4me3 (Albert et al., 2017). As a consequence, the inhibition of 1C metabolism in aRG would reduce their proliferation and abundance (Fawal et al., 2018).

Amino Acid Metabolism and Microcephaly

Several reports have described links between impaired amino acid metabolism and microcephaly. One study showed that mutations in *ASNS*, a gene encoding asparagine

synthetase, which converts aspartate and glutamine to asparagine and glutamate, cause microcephaly (Ruzzo et al., 2013). This finding is consistent with the importance of glutaminolysis for NPC proliferation, and raises the possibility that asparagine synthesis also has a role in this process. Another study showed that microcephalic patients carry mutations in genes which encode critical enzymes in the serine synthesis from 3-phosphoglycerate, an intermediate metabolite of glycolysis (Acuna-Hidalgo et al., 2014). These two studies therefore are consistent with an importance of anabolic processes in human fetal NPC proliferation (**Fig. 3**).

Concluding Remarks: Perspectives of Metabolic Regulation of Neocortical Expansion in Development and Evolution

Recent culumative evidence strongly suggests that certain pathways of cell metabolism, notably of mitochondrial metabolism, underlie increased neocortical NPC proliferation, and thus are a driving force of neocortical expansion in human evolution. In line with the growing importance of mitochondrial metabolism during neurogenesis, a very recent report (Iwata et al., 2020) has addressed the morphological plasticity of mitochondria during the early stages of cortical neuron differentiation. Yet, despite this progress, there are several big open questions. One concerns the functional consequences of the specific metabolic pathways for NPC proliferation. We have briefly discussed above how glycolysis ending at lactate and glutaminolysis may be beneficial for NPC proliferation, that is, by allowing an increase in various anabolic processes. However, that a "three-quarter TCA cycle" metabolic pathway (Fig. 3) indeed promotes cell cycle progression by supporting anabolic processes still needs to be corroborated. To this end, it will be crucial to perform extensive metabolomics and to obtain insight into causal relationships. Specifically, metabolic flux analyses using stable isotope-labled metabolites, such as C-13 glutamine (Antoniewicz, 2018), may allow us to trace the active metabolic pathway and directly examine whether a three-quarter TCA cycle contributes to anabolic processes.

Also, it will be important to determine whether changes in metabolism influence cell cycle length or the cell division mode (symmetric vs. asymmetric) of NPCs, or neuronal cell type-specific lineage patterns of neurogenesis, as these features are thought to change during evolution (Huang et al., 2020; Mora-Bermudez et al., 2016; Pollen et al., 2015). In order to answer these questions, expression analyses of critical metabolic enzymes will not be sufficient. Rather, it will be important to gain a comprehensive overview about the entire

metabolic activities in the different NPC subtypes in selected species, using state-of-the-art metabolomic approaches such as metabolic flux analyses. As shown in **Table 1**, for example, the metabolic activities of aRG and bRG with regard to glycolysis and glutaminolysis are somewhat similar. However, the activities of other metabolic pathways have not yet been examined in sufficient detail. Hence, our understanding of NPC metabolism is still far from complete.

Another key question concerns the sources of the metabolites that fuel the metabolic network specific to human neocortical NPCs. Since fetal metabolism largely depends on maternal metabolism, fetal NPC metabolism may be affected by the maternal metabolism. For example, recent studies have shown that the maternal metabolism affects (i) mitochondrial metabolism in mouse embryonic liver (Bowman et al., 2019) and (ii) mitochondrial mobility in NPCs as well as the thickness of the mouse embryonic neocortex (Rash et al., 2018). In addition, a maternal influence on the development of the mouse embryonic neocortex has very recently been reported (Stepien et al., 2020). Therefore, it appears worthwhile to study the maternal – fetal metabolic relationship in the context of neocortical development, its disorders, and neocortical evolution.

During human evolution, humans have utilized diverse sources of nutrients (Wrangham, 2013). An adaptation to the intake of food enriched in fat, protein or carbohydrate may have enabled human neocortical NPCs to take advantage of certain nutrients for their expansion by modulating cell metabolism. Thus, understanding NPC metabolism may be a good starting point for connecting evolutionary changes in feeding behavior and the evolutionary expansion of the human neocortex**.**

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Figures and Figure Legends

Figure 1. NPCs in neocortical development and evolution

(A) Cartoons of human fetal (top left, 11 weeks post conception) and adult (top middle) brain (cerebrum) and mouse embryonic (bottom left, embryonic day 16.5) and adult (bottom middle) brain and of coronal sections of the latter (right). Blue area indicates the gray matter. Scale bar (5 cm) applies to the brains, and scale bars of 10 mm and 1 mm apply to human and mouse coronal sections, respectively.

(B) Principal NPC types in developing neocortex of mouse and human. Orange areas indicate the subventricular zone (SVZ) in mouse and inner SVZ (ISVZ) and outer SVZ (OSVZ) in human. Pink, green and blue cells indicate APs, BPs and neurons, respectively. Distinct morphotypes of bRG are depicted. CP: cortical plate, IZ: intermediate zone, SP: subplate.

Fugure 2. Metabolic network in NPCs

Different metabolic pathways are color-coded as follows: turquoise, glycolysis; blue, β-oxdation; brown, cholesterol metabolism; orange, 1C metabolism; purple, glutaminolysis; green, TCA cycle. The mitochondrial compartment is indicated by light green. Proteins discussed in the text are indicated in red.

10 fTHF: 10-Formyltetrahydrofolate, 3-PHP: 3-phosphohydroxypyruvate, 5 mTHF: 5-Methyltetrahydrofolate, 5,10 mTHF: 5,10-Methylenetetrahydrofolate, αKG: α-ketoglutarate, CoA-SH: coenzyme A, Fructose 1,6P_2: fructose-1,6-bisphosphate, Fructose 6P: fructose 6-phosphate, GA3P: glyceraldehyde 3-phosphate, Glc: glucose, Glc 1P: glucose 1-phosphate, Glc 6P: glucose 6-phosphate, OAA: oxaloacetate, PEP: phosphoenolpyruvic acid, PSer: 3-phosphoserine, SAH: S-adenosylhomocysteine, SAM: S-adenosylmethionine, THF: tetrahydrofolate.

Figure 3. Three-quarter TCA cycle concept

Different metabolic pathways are color-coded as follows: turquoise, glycolysis; blue, fatty acid synthesis; brown, cholesterol synthesis; red, amino acid and nucleotide synthesis; magenta, glutaminolysis; green, three-quarter TCA cycle. The mitochondrial compartment is indicated in light green. αKG: α-ketoglutarate, CoA-SH: coenzyme A, Glc: glucose, OAA: oxaloacetate, PEP: phosphoenolpyruvic acid.

Table 1. Summary of metabolic pathway activities in mouse and human NPCs.

Table 1. Summary of metabolic pathway activities in mouse and human NPCs.

	APs		BPs				References
	aRG		bIPs		bRG		
	Mouse	Human	Mouse	Human	Mouse	Human	
Glycolysis	high	N.D.	low	N.D.	N.D.	N.D.	Khacho et al., 2016
Glutaminolysis	high	high	low	N.D.	N.D.	high	Journiac et al., 2020; Namba et al., 2020
β-Oxidation	high	N.D.	low	N.D.	N.D.	N.D.	Xie et al., 2016; Knobloch et al., 2017
Fatty acid synthesis	low	high	low	N.D.	N.D.	N.D.	Bowers et al., 2020
Cholesterol metabolism	high	N.D.	low	N.D.	N.D.	N.D.	Saito et al., 2009; Driver et al., 2016
1C metabolism	high	N.D.	low	N.D.	N.D.	N.D.	Fawal et al., 2018

The Table lists the extent of the activity of the indicated metabolic pathways either as deduced from changes in NPC abundance upon manipulation of the respective metabolic activity, or as deduced from metabolic activity measurements. Note that (i) the current

knowledge for human NPCs is less than that for mouse NPCs, and (ii) the metabolic regulation of behavior and fate of mouse NPCs is only partially understood.

N.D., no data

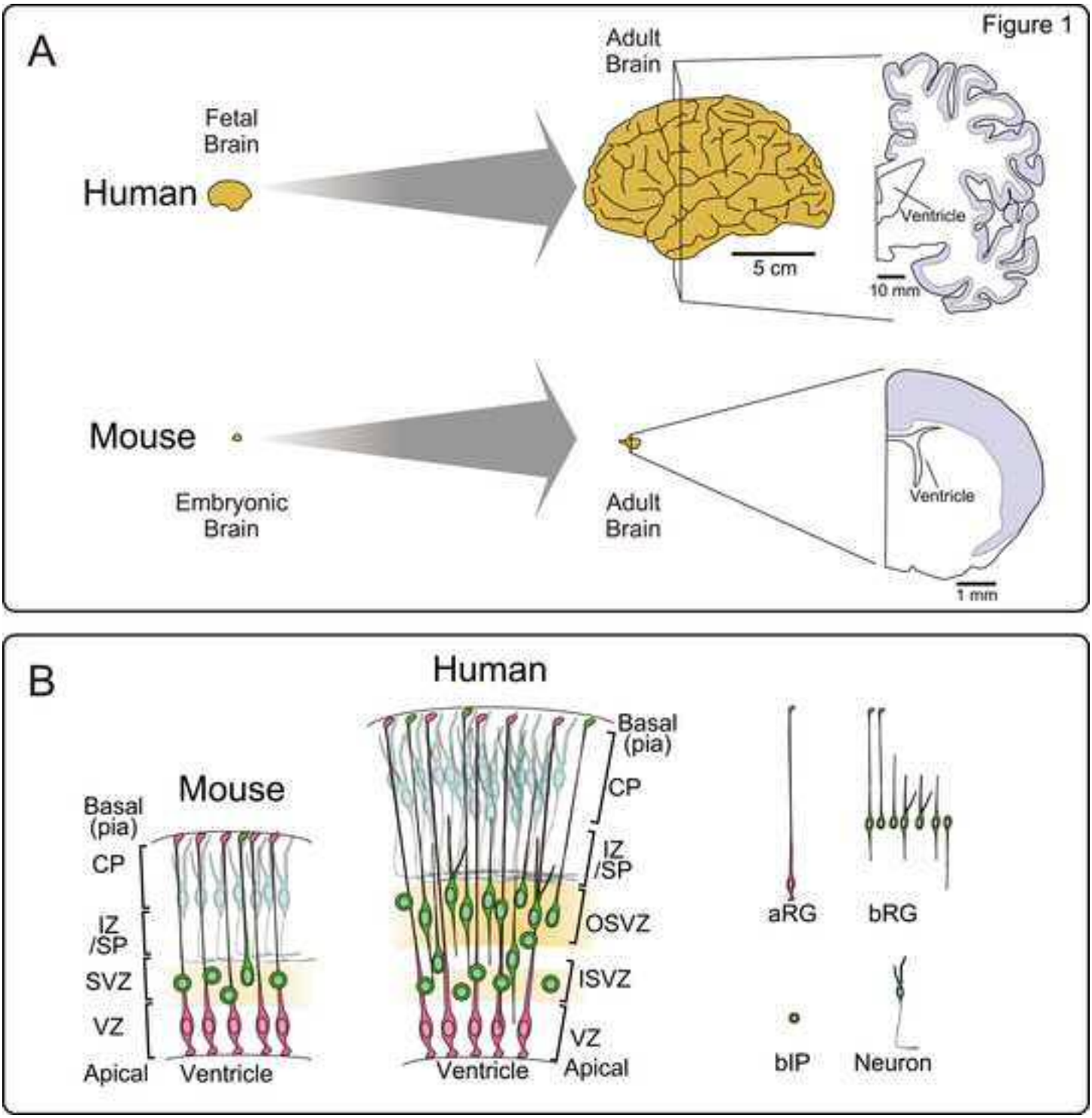

Figure 2

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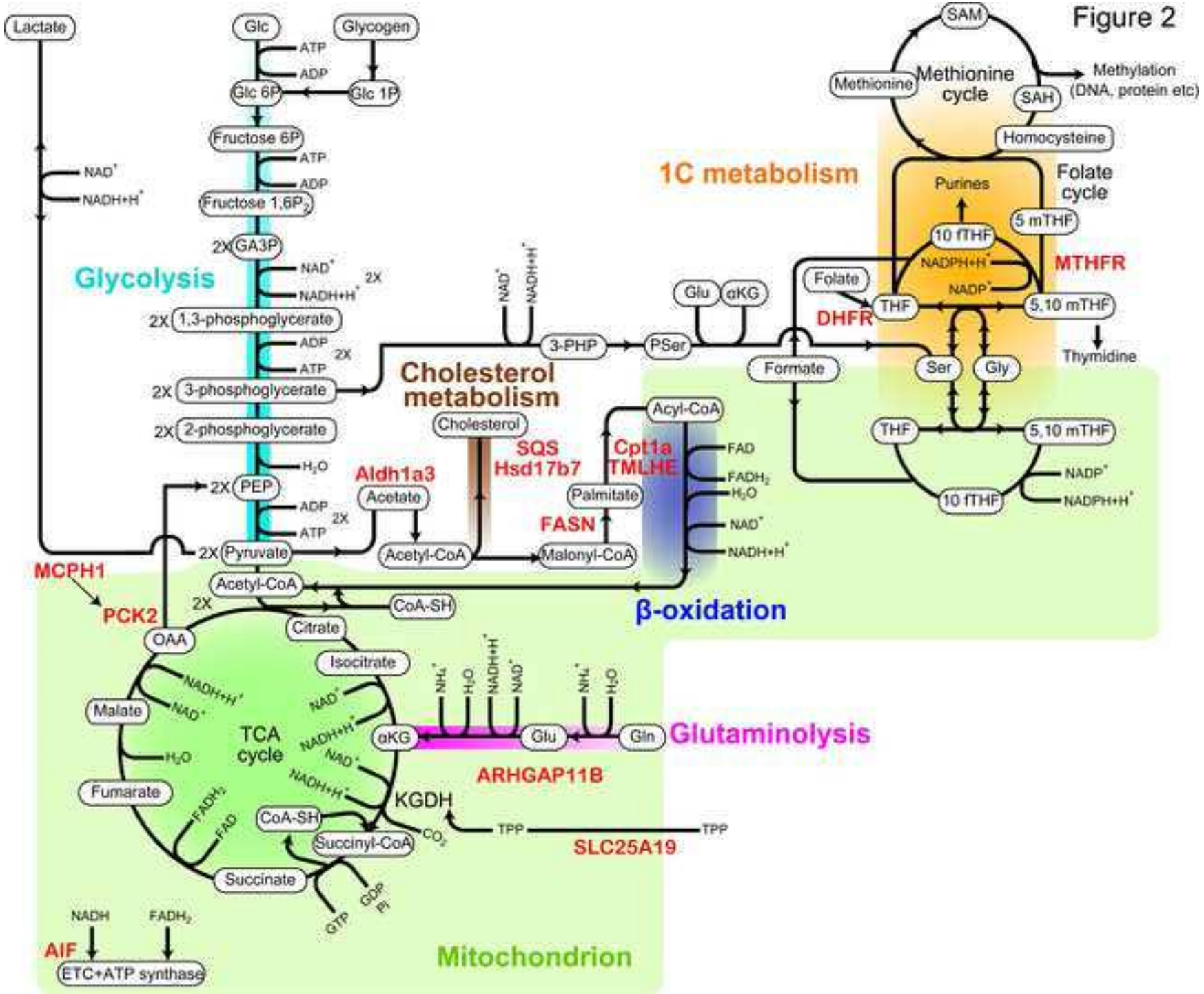

Figure 3

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