Review

Glial metabolism versatility regulates mushroom body–driven behavioral output in *Drosophila*

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Providing metabolic support to neurons is now recognized as a major function of glial cells that is conserved from invertebrates to vertebrates. However, research in this field has focused for more than two decades on the relevance of lactate and glial glycolysis for neuronal energy metabolism, while overlooking many other facets of glial metabolism and their impact on neuronal physiology, circuit activity, and behavior. Here, we review recent work that has unveiled new features of glial metabolism, especially in *Drosophila*, in the modulation of behavioral traits involving the mushroom bodies (MBs). These recent findings reveal that spatially and biochemically distinct modes of glucose-derived neuronal fueling are implemented within the MB in a memory type–specific manner. In addition, cortex glia are endowed with several antioxidant functions, whereas astrocytes can serve as pro-oxidant agents that are beneficial to redox signaling underlying long-term memory. Finally, glial fatty acid oxidation seems to play a dual fail-safe role: first, as a mode of energy production upon glucose shortage, and, second, as a factor underlying the clearance of excessive oxidative load during sleep. Altogether, these integrated studies performed in *Drosophila* indicate that glial metabolism has a deterministic role on behavior.

The brain is a highly energy-expensive organ and requires a constant input of energy to maintain neuronal activity and homeostasis. In an ad libitum–fed animal, the main energy source for the brain is glucose (Sokoloff 1999), which can fuel tasks either through the glia-mediated regulation of energy supply or via its direct utilization by neurons. Since the initial drafting of the astrocyte–neuron lactate shuttle (ANLS) as a mechanism for fueling activated glutamatergic synapses in mammals (Pellerin and Magistretti 1994), it took almost three decades to establish that a major function of glial cells, and astrocytes in particular, is to fuel neuronal activity on demand. A wealth of studies in mammals has concentrated on the tight neuron–glia coupling mechanisms that result in lactate delivery to neurons upon activation or during memory formation (Suzuki et al. 2011; Pellerin and Magistretti 2012; Barros 2013; Bonvento and Bolaños 2021). Several recent works in *Drosophila* have studied global phenotypes of survival or intensive thermogenetic neuronal activation, also reporting a critical role of glycolysis-derived lactate from glia (Volkenhoff et al. 2015; González-Gutiérrez et al. 2020). Thus, despite still-ongoing debate and controversies (Dienel 2017; Bak and Walls 2018; Yellen 2018), bolstered by parallel experimental evidence that neuronal glycolysis can also support brain activity (Bak et al. 2009; Ashrafi et al. 2017; Díaz-García et al. 2017), it can be stated that a major, conserved role of glial cells across phyla is to comply with neuronal energy needs.

This scientific debate has primarily remained focused on which cell type is the primary consumer of glucose in the brain, and whether lactate is indeed a relevant fuel for neurons. However, this overlooks the great versatility of glial metabolism and its multiple, diverse effects on neuronal physiology, which have only started to be unveiled in recent years, both in *Drosophila* (Liu et al. 2015, 2017; De Tredern et al. 2021; Goodman and Bellen 2022; Silva et al. 2022; McMullen et al. 2023; Rabah et al. 2023a,b; Raun et al. 2023) and in mammals (Bonvento and Bolaños 2021; Morant-Ferrando et al. 2023). The aim of this review is to describe how the emerging field of glial cell studies in *Drosophila* has made this organism a pioneer in characterizing glial metabolic versatility, leveraging the possibility of cell type–specific acute inactivation of targeted metabolic pathways in vivo, in connection with specific behaviors involving defined neural circuits. In the first part, we focus on the diversity of metabolic coupling schemes between glial glycolysis and neuronal mitochondrial metabolic activity, and how their spatial organization impacts behavior. In the second part, we describe how glial glucose and lipid metabolism are both involved in managing the neuronal levels of reactive oxygen species (ROS). Finally, we discuss the role of glial lipid oxidation in situations of nutritional shortage or increased sleep need.

Glial cells in the adult fly brain

Because several previous reviews have already described in detail the anatomy and main functional attributions of *Drosophila* glia cells (Freeman 2015; De Backer and Grunwald Kadow 2022), we briefly summarize here the different glial cell types that have been defined. The adult *Drosophila* brain contains five types of glial cell populations, which are named after their spatial arrangement or their association with neurons (Fig. 1; Awasaki et al. 2008; Freeman 2015; Ou et al. 2016; Kremer et al. 2017). The perineural and subperineural glia together comprise the hemolymph–brain barrier, equivalent to the mammalian blood–brain barrier (Stork et al. 2008; Desalvo et al. 2011; Schirmeier and Klämbt 2015; Weiler et al. 2017). The cortex glia lie in close proximity to the soma, or cell bodies, of neurons (Freeman and Doherty 2006) and form a mesh-like structure around them, with each glial cell often enwrapping multiple (up to 100) neuronal cell bodies (Awasaki et al. 2008; Kremer et al. 2017). To some extent, they can be compared to mammalian astrocytes because of their spatial proximity to both the brain barrier and neurons, suggesting they could be involved in nutrient exchange and barrier permeability regulation

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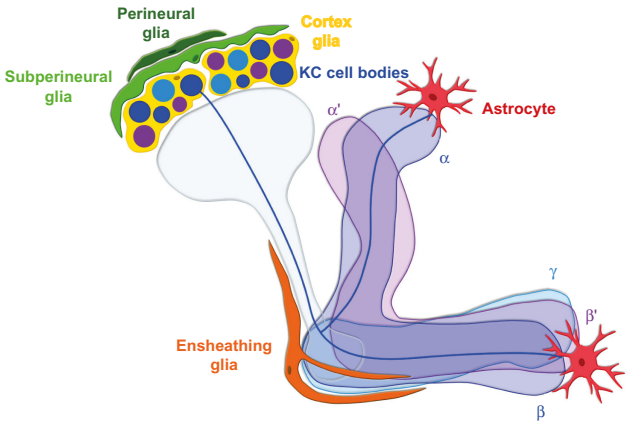

Figure 1. Anatomy and localization of the five types of glial cells in the *Drosophila* central brain illustrated on the mushroom body (MB). The MB is a bilaterally symmetrical structure in the adult *Drosophila* brain. The cell bodies of the intrinsic MB neurons, called Kenyon cells (KCs), cluster on the posterior part of the brain. The axons project toward the anterior part of the brain and ramify dorsally to form the vertical α and α′ lobes, and medially to form the horizontal β, β′, and γ lobes (Tanaka et al. 2008). Five main types of adult glia are shown. Together, the perineural and subperineural glia form the surface glia. The cortex glia surround the neuronal cell bodies. Ensheathing glia surround the neuropil and separate the distinct MB lobes. Finally, astrocyte-like glia (ALG) branch into the axons and are associated with the synapses (Awasaki et al. 2008; Freeman 2015; Ou et al. 2016; Kremer et al. 2017).

(Abbott et al. 2006; Artiushin and Sehgal 2020), as well as metabolic supply to neurons (see next sections). The brain neuropil contains two types of glia: fibrous ensheathing glia and branching astrocyte-like glia (ALG). Ensheathing glia encase axon bundles to isolate neuronal compartments (Awasaki et al. 2008), are involved in phagocytosis during neurotransmitter clearance and after brain injury (Doherty et al. 2009), and were recently found to regulate aging and its physiological indicators (Sheng et al. 2023). ALG distinctively resemble mammalian astrocytes in form and function (Stork et al. 2014; Freeman 2015). These glia innervate the neuropil in a tiling manner (Pogodalla et al. 2022); are associated with synapses, with their proliferation inversely dependent on the synaptic density (Kremer et al. 2017); and are heavily involved in neuronal remodeling and axonal pruning (Boulanger and Dura 2022; Corty and Coutinho-Budd 2023). Altogether, despite making up only ∼10% of the total fly brain cellular mass (Kremer et al. 2017), the various glia types are crucial in maintaining morphological integrity, neurotransmitter homeostasis, and physiological health for a functioning nervous system. Glia are also deeply involved in assisting with the proper development of neurons during embryogenesis and adult formation (Ou et al. 2014; Yang et al. 2021; Marmor-Kollet et al. 2023); however, we have restricted our review to the role of glia in the adult central brain.

The *Drosophila* mushroom body (MB), in particular, has been an important brain area for studying how glial metabolism is dynamically regulated and articulated within various adaptive or homeostatic behaviors, such as learning and memory and sleep (Joiner et al. 2006; Pitman et al. 2006; Modi et al. 2020). The GAL4/UAS system of spatially controlled genetic manipulation (Brand and Perrimon 1993) has been instrumental in the anatomical and functional dissection of neuronal circuits down to the cellular resolution. In particular, the generalization of intersectional strategies for restricting the expression pattern of genetic driver lines (Aso et al. 2014) has greatly improved single-cell (or single-cell type) targeting. In comparison to the highly sophisticated tools available for neuronal studies, the region specificity of genetic targeting strongly limits glial studies. Although genetic driver lines have been identified that target each of the distinct glial cell categories in the whole nervous system, region-specific tools that would allow spatially restricted targeting of a given glial type—for instance, astrocytes in contact with MB neurons—are currently missing (Kremer et al. 2017). This might indicate that individual glial cells lack a precise genetic identity, contrary to neurons (Davie et al. 2018), and are instead defined by their local interaction with neurons. Still, this apparent lack of genetic discrimination within distinct glial cells of the same category complicates the spatial attribution of observed behavioral phenotypes. Thus, even when studying MB-related behavior, it remains generally difficult to ascribe a specific role to the glia in the MB area. In this review, we, therefore, describe studies revealing glial involvement in MB-related functions, even if in some cases the MB–glia interaction has not been strictly established. Experimental approaches combining behavior assays and imaging techniques, in which physiological monitoring can be focused on a specific brain area, can help overcome this technical obstacle and currently represent perhaps the most valuable strategy for the fine analysis of neuron–glia coupling in relation to behavior, at least until transformative cell-targeting approaches can provide glia studies with the same level of sophistication as neuronal studies.

Glial cells provide glucose-derived energy to MB neuronal mitochondria for memory formation

Olfactory learning and memory are the principal functions sustained by the MB neuronal assembly, which has been extensively studied using the well-established classical olfactory associative conditioning paradigm (Tully and Quinn 1985), in particular, regarding the role of fly glia in memory. Aversive olfactory conditioning involves the presentation of one odor paired with electric shocks followed by another odor without the negative stimulus (Tully and Quinn 1985; Tully et al. 1994). Flies can form distinct types of olfactory memory that differ genetically and functionally, ensuring that different behavioral outputs are derived from discrete storage and retrieval circuits (Bouzaiane et al. 2015). A single cycle of training can yield an avoidance memory that lasts for hours. This memory can be subdivided into sequential phases. Immediate short-term memory (STM) involves γ Kenyon cells (KCs) and downstream MB output neurons (Blum et al. 2009; Bouzaiane et al. 2015; Owald et al. 2015), whereas in the ∼3-h time window after conditioning, memory behavior results from the parallel retrieval of a labile middle-term memory (MTM) component encoded in and retrieved from α/β KCs, and another memory component, called anesthesia-resistant memory (ARM) because of its resistance to cold-shock anesthesia, that involves the same networks as STM (Bouzaiane et al. 2015). One day after a single cycle of training, learned avoidance behavior is strongly dampened. However, the time-spaced repetition of training cycles (i.e., spaced training) allows memory consolidation into long-term memory (LTM), which can persist up to 1 wk and depends on de novo protein synthesis, unlike the less persistent memory formed after massed training which typically decays within 1–2 d (Tully et al. 1994).

Regular brain activity incurs high energy costs (Magistretti et al. 1999), and memory formation is no exception (Mery and Kawecki 2005; Plaçais and Preat 2013; Plaçais et al. 2017; Padamsey and Rochefort 2023). The recent development of genetically encoded sensors for various carbohydrate derivatives (San Martín et al. 2014) has provided access to cell type–specific measurement of the dynamics of glucose metabolic fluxes after olfactory conditioning. An experimental approach combining two-

photon in vivo imaging of a pyruvate FRET sensor (San Martín et al. 2014) with acute pharmacological disruption of the mitochondrial respiratory chain has proven a powerful strategy to probe the metabolic activity of KC mitochondria, through the rate of their pyruvate uptake (Plaçais et al. 2017; Rabah et al. 2023a; Comyn et al. 2024). The observation that knocking down MPC1, the mitochondrial pyruvate transporter, in all KCs fully impairs MTM after single-cycle training and LTM after spaced training, while leaving ARM intact after single-cycle training or massed training, raised the possibility of memory type–specific regulation by glial cells. Remarkably, the spatiotemporal reorganization of metabolic activation that was shown to occur between single and spaced training is reflected at the level of the glia cells: Although in both cases the increased metabolic activity in KCs is supported by glial cells, distinct organizations of neuron–glia metabolic coupling are implemented depending on the conditioning protocol.

A single training cycle elicits an acute increase in pyruvate import into neuronal mitochondria, which lasts no more than 2 h and is critical for MTM (Rabah et al. 2023a; Comyn et al. 2024). This effect has been reported in the KC vertical lobes and in the soma region (Rabah et al. 2023a), indicating whole-cell metabolic activation. The cortex glia sustain this KC metabolic enhancement through a compartmentalized oxidation of glucose between the cortex glia and KCs (Rabah et al. 2023a). Glucose is imported in the cortex glia through a specific transporter called "glucose uptake by glia" (glug). There, it is broken down into pyruvate through glycolysis, followed by conversion into L-alanine by the ALAT (alanine transaminase) enzyme. This L-alanine is shuttled to the MB neurons, where it is reconverted into pyruvate to subsequently fuel the mitochondrial oxidative phosphorylation that sustains MTM. Increased pyruvate consumption in MB vertical lobes and in somas relies on the activity of ALAT or the glycolytic enzyme phosphofructokinase (PFK) in cortex glia, showing that the cortex glia alone can support the whole-KC-wide metabolic plasticity after a single-cycle training (Fig. 2A; Rabah et al. 2023a).

The spaced repetition of training cycles, which lifts the default inhibition of LTM formation, also results in increased mitochondrial pyruvate uptake, contrary to massed training (Plaçais et al. 2017). However, this effect differs in time and space from that observed after single-cycle training: It persists for at least 8 h after the end of spaced training (although it stops after 24 h, when LTM retrieval is typically tested), but only in the axonal compartment, because the duration of the metabolic activation in the somas does not exceed that observed after single-cycle training (Comyn et al. 2024). Although ALAT knockdown in astrocytes did not affect memory performance after single-cycle training, it impaired LTM after spaced training and increased pyruvate uptake by mitochondria in the MB vertical lobes (Rabah et al. 2023a). In the case of spaced training, up-regulated energy metabolism is not only necessary, it is also sufficient to trigger LTM formation, as the pre-stimulation of mitochondria pyruvate uptake facilitated LTM formation (Plaçais et al. 2017). The long-lasting metabolic plasticity in MB axons that gates LTM formation is, therefore, accompanied by and necessitates the recruitment of astrocytes, to locally fuel axonal mitochondria in place of cortex glia (Fig. 2B).

Overall, studies of the metabolic underpinnings of olfactory memory have revealed the specialization of distinct glial cell subtypes for different memory phases (e.g., the cortex glia for MTM and astrocytes for LTM). It should be noted, however, that all genetic manipulations impairing alanine transfer from cortex glia to neurons also impaired LTM in addition to MTM, further suggesting that LTM derives from the consolidation of a memory trace underlying MTM. Although they involve distinct types of glial cells, MTM and LTM both rely on the compartmentalization of glucose metabolism between KCs and glia, strongly reminiscent of the ANLS scheme of metabolite transfer documented in mammals (Magistretti et al. 1999; Pellerin and Magistretti 2012; Magistretti and Allaman 2015). Although in mammals lactate transfer is instrumental in memory establishment through an astrocyte–neuron dialogue (Newman et al. 2011; Suzuki et al. 2011; Descalzi et al. 2019), *Drosophila* MBs notably differ in that alanine, rather than lactate, mediates the glia–neuron metabolic coupling, at least for memory types whose formation relies on α/β KCs. Nevertheless, recent evidence does suggest that lactate could be relevant to sustaining other memory processes in distinct KC subsets.

Lactate is interconvertible with pyruvate through the enzyme lactate dehydrogenase (Ldh), with multiple isoforms distributed among glial populations present in mammalian brains (Bittar et al. 1996; Laughton et al. 2007); however, only a single form is present in *Drosophila* (Onoufriou and Alahiotis 1982). Recently, lactate homeostasis was implicated in aging and neurodegeneration contexts (Long et al. 2020). Within the KCs, Ldh expression is restricted and enriched in MB γ neurons (Jones et al. 2018; Raun et al. 2023). Compared to aversive olfactory memory, courtship memory protocol involves learning displayed by males upon repeated rejection of courtship attempts by previously mated females. Courtship LTM is also consolidated in MB neurons, specifically the γ KCs (McBride et al. 1999; Montague and Baker 2016). As for olfactory aversive LTM, the MB knockdown of pyruvate dehydrogenase phosphatase and citrate synthase, two enzymes related to mitochondrial oxidative phosphorylation, impair courtship LTM, but not STM. Ldh knockdown results in similar phenotypes, and Ldh activity appears to be related to the maintenance of intracellular pyruvate levels (Raun et al. 2023), consistent with Ldh activity providing pyruvate in metabolic support of courtship LTM formation.

Astrocyte-derived lactate plays a critical role in avoidance LTM in rats (Suzuki et al. 2011). Although in *Drosophila* the source of lactate for courtship LTM is unknown, a glial origin can be speculated by analogy with mammals and by considering the fact that *Drosophila* glial cells can produce lactate from glucose (Volkenhoff et al. 2015). Furthermore, *Drosophila* Ldh levels naturally increase upon aging (Long et al. 2020), and in old males, glial down-regulation of Ldh, as well as its neuronal up-regulation or down-regulation, all disrupt long-term courtship memory (Frame et al. 2023). Whether Ldh activity and glycolysis are also required in selected subtypes of glial cells for courtship in young adults still remains to be directly tested.

These recent insights into courtship memory suggest that regardless of the type of KC involved, both MTM and LTM involve glial metabolic support, although α/β and γ KCs could differ in their use of alanine or lactate as a metabolic shuttle, respectively. Nevertheless, the molecular and cellular origins of this distinction remain unexplored.

Glial metabolic support to neurons is an integral component of memory formation, and it is important to consider how alterations in glial metabolic pathways can impact memory, particularly during aging. Intriguingly, the glial metabolic enzyme pyruvate carboxylase (PCB) was reported to be critically involved in age-related memory impairments (AMIs) in *Drosophila* (Yamazaki et al. 2014). PCB expression increases in glial cells during aging, and glial knockdown of PCB could mitigate AMI. PCB-induced AMI has been linked to a decrease in D-serine availability, the main coagonist of NMDA receptors. Glutamate release from ensheathing glia was recently reported to signal punishing stimuli to KCs during learning (Miyashita et al. 2023), suggesting that decreased D-serine could provide a sensory-based mechanistic explanation to AMIs. Alternatively, increased PCB activity in the cortex glia could consume pyruvate and decrease its availability for conversion to alanine (or lactate) and further export to KCs. As serine can be readily converted into pyruvate, this scenario is also compatible with the reported observation that supplementing

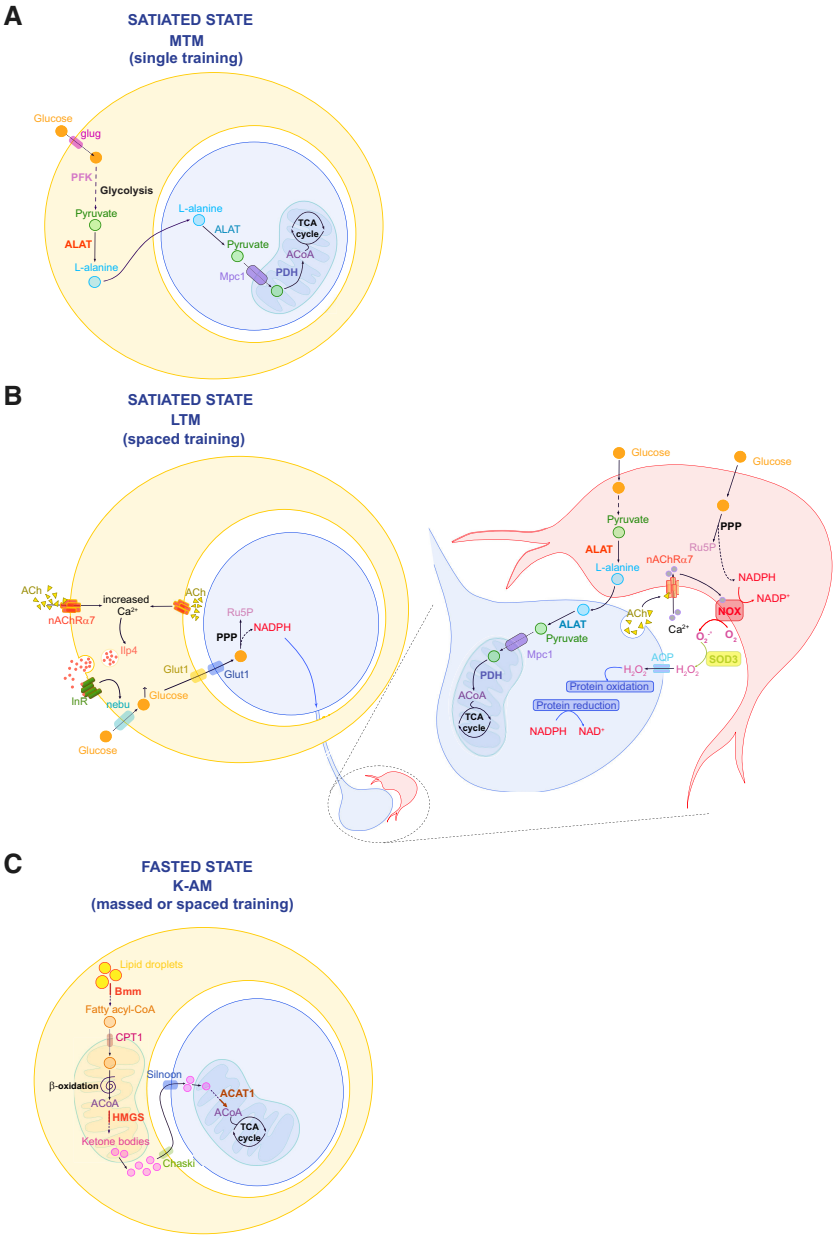

Figure 2. Different memory phases involve distinct modes of metabolic recruitment of glial cells by Kenyon cells (KCs). Similar to Figure 1, cortex glia are shown in yellow, astrocyte-like glia (ALG) are depicted in red, and KCs are in blue. (*A*) A single cycle of associative aversive conditioning leads to the formation of middle-term memory (MTM). The cortex glia import glucose via glug transporters; glucose is then glycolyzed into pyruvate, which in turn is converted into L-alanine by the alanine transaminase (ALAT) enzyme. This L-alanine is transported into the mushroom body (MB) neurons at the level of the soma, where it is reconverted into pyruvate. It then enters the mitochondria through the Mpc1 transporter and is broken down into acetyl-CoA (ACoA) by pyruvate dehydrogenase (PDH), which fuels the mitochondrial tricarboxylic acid (TCA) cycle to produce energy for MTM formation through oxidative phosphorylation (Rabah et al. 2023a). (*B*) Spaced training drives energy-expensive long-term memory (LTM) consolidation (Plaçais et al. 2017). ALAT-mediated conversion of pyruvate into L-alanine occurs in the ALG surrounding the local KC axons, where it is involved in up-regulating neuronal mitochondrial oxidative phosphorylation (Rabah et al. 2023a). Spaced training also triggers cholinergic excitation of cortex glia via the action of acetylcholine (ACh) on α7 nicotinic receptor (nAChRα7), which induces the release of insulin-like peptide 4 (Ilp4) and the autocrine stimulation of the insulin receptor (InR) in the cortex glia. This results in increased glucose uptake through nebu, a separate type of cortex glia glucose transporter, which is shuttled into the neurons via their Glut1 transporters. This glucose fuels the pentose phosphate pathway (PPP) to produce molecules required for gene expressions, such as ribulose-6-phosphate (used in nucleotide biosynthesis) and NADPH (a reducing agent) (De Tredern et al. 2021). At the axonal level, cholinergic activation of astrocytic nAChRα7 results in a Ca^{2+} surge; along with NADPH sourced from astrocytic PPP, this activates the NADPH oxidase (NOX) enzyme, finally producing $O_2^{\circ-}$. This $O_2^{\circ-}$ is converted into H_2O_2 by extracellular superoxide dismutase 3 (SOD3) expressed by ALG. H_2O_2 is imported into the α/β lobes via aquaporin (AQP) and drives the oxidation of proteins involved in a redox signaling cascade, resulting in LTM formation. NADPH produced at the cortex glia level travels to the axons and also participates in protein reduction (Rabah et al. 2023b). (*C*) Starvation induces the formation of a unique type of consolidated memory called ketone body (KB)-dependent aversive memory (K-AM) (Silva et al. 2022). Lipid droplet stores of cortex glia are mobilized into fatty acids (FAs) by Brummer (Bmm) lipase activity. FAs are imported into mitochondria via the CPT1 transporter, where they undergo β-oxidation into ACoA, which is subsequently catalyzed by the HMGS enzyme to produce KBs. These KBs are exported from cortex glia through the Chaski transporters and imported into MB neurons via the Silnoon transporters. They then enter the neuronal mitochondria where they are converted by the ACAT1 enzyme into ACoA, which enters the TCA cycle.

D-serine could rescue AMIs (Yamazaki et al. 2014). The glial cell subtypes in which PCB expression increases to elicit AMIs have not been investigated, although this may help illuminate the processes through which alterations in glial metabolism impair memory in the course of aging.

Altogether, a body of recent work has highlighted a critical role for both cortex glia and ALG in sustaining KC metabolic needs, and therefore in memory formation. Nevertheless, energy supply is more than a platform for neuronal signaling, as KC metabolic plasticity can regulate memory dynamics in itself. Thus, depending on the learning context, the proper implementation of specific glia–neuron metabolic schemes should be considered an integral part of MB-relevant cognitive processes.

Glial cells both create and buffer ROS in KCs

ROS are major by-products of cellular energy metabolism. Given the prominent role of both cortex glia and ALG in sustaining mitochondrial activity in KCs, it should come as no surprise that these glia are also involved in maintaining the redox balance of KCs. ROS is a comprehensive term referring to numerous oxidant molecules such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). The primary and most widely documented effect of these highly reactive molecules is to induce cellular oxidative damage, which is why ROS levels are strictly regulated by scavenging enzymes (Murphy et al. 2011; Sies and Jones 2020). In the cell, mitochondrial activity is primarily responsible for ROS generation, and neurons are particularly susceptible to this because of their physiological dependence on mitochondrial energy production (Johri and Beal 2012). However, ROS are also important signaling molecules in favorable biological processes such as cell growth, survival, and stress response (Sies and Jones 2020), in which they typically trigger the reversible oxidation of redox-sensitive cysteine residues on downstream proteins, causing them to undergo structural (and thereby functional) changes (Lambeth and Neish 2014). These reports demonstrate that the functional ambivalence of ROS has long been recognized. Recent work in *Drosophila* has clarified that distinct glial cell types are involved in leveraging the positive role of ROS in memory-relevant signaling and in buffering the potentially harmful consequences of ROS production in KCs.

In the brain, ROS signaling is implicated in synaptic plasticity and long-term potentiation (Bindokas et al. 1996; Knapp and Klann 2002; Massaad and Klann 2011). Recent work from our team has provided in vivo evidence that ROS signaling contributes to memory formation in *Drosophila* (Rabah et al. 2023b). By exploiting the aforementioned aversive olfactory memory paradigm, this study established that LTM formation following spaced training specifically relies on ROS signaling in KCs. At the cellular level, in vivo imaging experiments using an ultrasensitive H_2O_2 fluorescent probe revealed a branch-specific import of H_2O_2 in the vertical lobes of α/β KCs through aquaporin channels after training. H_2O_2 triggers a cascade of oxidation in redox relay proteins crucial for LTM formation, most likely through the activation of redox-sensitive signaling pathways that remain to be identified. Unexpectedly, H_2O_2 was provided by ALG, highlighting a new mechanism of plasticity-relevant glia–neuron coupling termed astrocyte–neuron H_2O_2 signaling (ANHOS). Acetylcholine activation, assumed to be released by KCs, acts on the astrocytic nicotinic receptor nAChRα7. The resulting Ca^{2+} surge activates NADPH oxidase (NOX) enzyme producing extracellular $O_2^{\bullet-}$. The final conversion of $O_2^{\bullet-}$ to H_2O_2 is controlled by extracellular superoxide dismutase 3 (SOD3) expressed by astrocytes. As for ALG, it is remarkable that ANHOS-sustaining NOX activity required ongoing NADPH production by the pentose phosphate pathway (PPP), one of the two main biochemical pathways of glucose catabolism along with glycolysis (Fig. 2B). As noted earlier, ALG also provided pyruvate-derived alanine to KCs after spaced training for LTM formation (Rabah et al. 2023a). Although alanine production in cortex glia after single-cycle training clearly originates from glycolysis-derived pyruvate, the role of glycolytic enzymes in ALG has not been assessed. It remains to be determined whether (1) PPP and glycolysis co-occur in ALG, thereby potentially competing for glucose; (2) PPP and glycolysis occur in different compartments or processes of a given ALG cell; (3) distinct ALG cells specialize in H_2O_2 production through PPP and alanine production through glycolysis, respectively, after spaced training; or (4) PPP alone produces both NADPH and glycolytic intermediates such as fructose-6-phosphate or glyceraldehyde-3-phosphate (Jiang et al. 2014), which could be in turn further converted into pyruvate.

The role of ALG in producing ROS for LTM formation in the MB vertical lobes is remarkably contrasted by a parallel role of cortex glia at the somatic level. Indeed, cortex glia are also subject to cholinergic activation through nAChRα7 after spaced training (De Tredern et al. 2021). This triggers the release of insulin-like peptide 4 (Ilp4) and the autocrine stimulation of the insulin receptor (InR) in the cortex glia, resulting in increased glucose uptake specifically mediated by the glucose transporter nebu (De Tredern et al. 2021; Rabah et al. 2023a). This process sustains the shuttling of glucose through Glut1 to the KCs, where it fuels the PPP (Fig. 2B; De Tredern et al. 2021). Glut1 knockdown in KCs resulted in increased intracellular H_2O_2 levels after spaced training. This indicates that PPP activation in KCs buffers the increase in the oxidation state that would otherwise be caused by H_2O_2 import from astrocytes (Rabah et al. 2023b), consistent with a significant role for PPP in protection against oxidative stress through NADPH production (Bolaños and Almeida 2010). Overall, aversive LTM formation after spaced training necessitates a transient oxidative state imbalance in KCs for signaling purposes. Glial cells are critical players in both creating this imbalance, through NOX activity in ALG, and buffering it, through cortex glia–derived glucose supply to the PPP in KCs. Interestingly, it was recently shown in mice that astrocyte β-oxidation promotes the generation of ROS, which plays a beneficial role in cognition (Morant-Ferrando et al. 2023), suggesting that the role of glia as producers of ROS could be conserved.

In other contexts than memory formation, several *Drosophila* studies have revealed another mechanism, involving lipid homeostasis, through which glia can offer protection against ROS-induced toxicity in cases when the neuronal ROS-buffering capacity is overloaded (Bailey et al. 2015; Liu et al. 2015, 2017). Although this research was performed in the neuroblasts of developing larval brains or in the adult fly retina, they are worth mentioning here, as the described mechanisms might enrich the antioxidant properties of cortex glia in the adult central brain, in particular in the MBs. Genetic mutations of the mitochondrial respiratory chain in neurons of the fly retina were used as a model of prolonged, elevated ROS production leading to neurodegeneration (Liu et al. 2015). In this context, neighboring glial cells responded to ROS by lipid droplet (LD) accumulation, because of the JNK pathway-mediated activation of the sterol regulatory element-binding protein (SREBP), a crucial mediator of lipid biosynthesis (Liu et al. 2015). LD accumulation is at least in part fed by lipid import from neurons. Indeed, in the context of an overloaded mitochondrial TCA cycle in which mitochondrial consumption of acetyl-CoA and of its precursor pyruvate is reduced, the import of glia-derived lactate and subsequent Ldh activity in neurons were shown to provide substrates for neuronal fatty acid synthesis (Liu et al. 2017). Impairing lipid transport from neurons to glia, which rely on apolipoproteins neuronal Lazarillo (NLaz) and glial Lazarillo (GLaz), accelerates neurodegeneration (Liu et al. 2017), suggesting that lipid export to glia is beneficial to neuronal

protection. However, the earlier report that preventing glial LD accumulation through overexpression of the lipase Brummer delays the onset of neurodegeneration somehow led to a contradictory conclusion that glial LD can promote neurodegeneration (Liu et al. 2015). Therefore, ROS-induced LD accumulation appears to serve a dual function. Support for the protective role of LDs has come from another study performed in larval neuroblasts (Bailey et al. 2015). In this study, the accumulation of LDs enriched with polyunsaturated fatty acids (PUFAs) in niche glia (including cortex glia and subperineural glia) provided protection against oxidative stress and enabled stem cell proliferation despite starvation (Bailey et al. 2015). PUFA peroxidation has an adverse effect on neuroblast production and not only drives up ROS production via a positive feedback loop, but also causes damage to other macromolecules through its interaction with peroxidative by-products such as 4-hydroxy-2-nonenal, which targets proteins. In this scenario, the relocation of PUFAs from the cell membrane, where it is more vulnerable to interactions with ROS, into the core of LDs prevents lipid peroxidation by making them less accessible to ROS-induced harm. Thus, it remains to be elucidated to what extent LD accumulation in glia is protective or detrimental in adult brains subjected to high ROS production. From the literature, it seems that lipids with ROS-induced damage are the most toxic when retained in neurons, and that glial storage provides a preferable, although perhaps not perfect, scenario. It is possible that the initial encapsulation of peroxidated FA by LD could represent a transient protection. Excessive storage of damaged lipids, though, would ultimately stall the neuron–glia lipid transfer system, as well as become toxic for glial cells themselves. In such a case, lipase overexpression could be an artificial way to enhance β-oxidation of fatty acids and consume damaged lipids.

Altogether, glial metabolism as a whole is tightly connected to the redox status of neurons through various neuron–glia coupling mechanisms. In healthy conditions, glucose uptake by astrocytes and cortex glia tunes and buffers neuronal ROS levels, respectively, whereas the use of lipid synthesis and storage machinery seems more devoted to handling excessive and potentially damaging levels of ROS. This critical role of LD in glial cells brings into question how glial cells manage their LD stores, and in particular the role of lipid catabolism in this process.

Glial lipid oxidation is a fail-safe metabolic state operating during fasting and sleep

During nutrient-deficient conditions such as starvation periods, organisms tend to shift from glucose to alternate energy sources in order to prioritize and ensure survival, either by recruiting previously stored glycogen (Wender et al. 2000; Brown et al. 2003; Herzog et al. 2008) or using ketone bodies (KBs) such as acetoacetate and β-hydroxybutyrate to produce acetyl-CoA that can enter the mitochondrial TCA cycle. In mammals, the main producer of KBs is assumed to be the liver (McGarry and Foster 1980). Ex vivo studies have shown that KBs are indeed capable of sustaining neuronal oxidative metabolism for basal housekeeping purposes (Chowdhury et al. 2014). However, until recently it was not known whether KBs could also support neural circuit activity and higher-order brain functions. LTM formation is an energy-costly process that shuts down when flies are fasted at the time of and after conditioning (Plaçais and Preat 2013). Although fasted flies are unable to form genuine LTM, they can still display robust massed or spaced memory performances 24 h after conditioning (Plaçais and Preat 2013). Recently, it was shown that KCs rely on KBs to support memory formation after associative aversive learning during nutrient deficiency (Silva et al. 2022). The results show that remarkably, at least in the context of memory formation, KBs were locally synthesized in the brain by cortex glia and delivered to KCs (Silva et al. 2022). Activation of AMPK, the master cellular energy sensor, by starvation stress triggered lipolysis of the LDs stored in glial cells, through the action of the lipase Brummer (Silva et al. 2022). The resulting FAs were translocated into the glial mitochondria as fatty acyl-CoA via CPT1 transporters to be processed via β-oxidation into acetyl-CoA, and further converted into KBs. The transfer of KBs from cortex glia to KCs is mediated by two distinct monocarboxylate transporters: Chaski in the cortex glia, and Silnoon in neurons (Fig. 2C).

This study thus revealed the ketogenic abilities of glia, and its physiological relevance in vivo as a "backup" mode of fueling memory formation in conditions of low glucose availability. In another context of defective glucose metabolism—namely, genetically impaired glycolysis in all glia cells—that is detrimental to neuronal survival (Volkenhoff et al. 2015), it was reported that glial β-oxidation and ketogenesis are also triggered, which slows down neurodegeneration (McMullen et al. 2023). Overall, these reports are consistent with lipid oxidation and ketogenesis in glia acting as a fail-safe mode of metabolic support to neurons, in the central brain in general in dysfunctional conditions, and in MB neurons in particular for memory formation.

Sleep is another behavior that has been recently linked to glial lipid metabolism, although not necessarily through interaction with KCs. Several manipulations linked to lipid homeostasis in distinct glial cell subtypes are reported to induce sleep phenotypes, based on mechanisms that are likely conserved across species. Sleep fragmentation was observed upon overexpression in fly astrocytes of a human fatty acid–binding protein 7 (FABP7) carrying a missense mutation identified as causing sleep fragmentation in humans (Gerstner et al. 2017). Another study recently showed that manipulating the hemolymph–brain barrier permeability (comprising perineural and subperineural glia) can lead to altered, increased trafficking, and buildup of acylcarnitines. This has the effect of enhancing sleep need (Li et al. 2023a), in line with previous observations of acylcarnitine accumulation in sleep-deprived mice (Hinard et al. 2012) and humans (Davies et al. 2014; Weljie et al. 2015).

Although these studies established links between lipid-relevant proteins or metabolites in glia and sleep, no mechanistic picture has emerged yet from these few reports in distinct glial subtypes. Deeper insight into the role of glial lipids in sleep regulation was provided by a more recent study that examined the role of ecdysone signaling in sleep regulation, which clearly identified a major role for glial cells in sleep regulation (Li et al. 2023b). Feeding flies with exogenous ecdysone increased their sleep, indicating a sleep-promoting effect of ecdysone. Brain immunostainings of the ecdysone receptor EcR revealed preferential expression in cortex glia, and EcR knockdown in this cell type specifically decreased total sleep. Interestingly, ecdysone signaling and sleep amount inversely correlated with LD content in cortex glia (Li et al. 2023b), consistent with a previously described role for ecdysone signaling in glial lipid mobilization in larvae (Kis et al. 2015). Based on these observations, it was proposed that ecdysone modulates sleep by stimulating lipid metabolism in cortex glia (Li et al. 2023b).

Perhaps related to this finding, it was recently reported that both wake state and sleep deprivation can induce an increased brain-wide mitochondrial oxidation state in glia but not in neurons, as measured by two different mitochondrial oxidation sensors, as well as LD accumulation specifically in cortex glia and ensheathing glia (Haynes et al. 2024). Knockdown of NLaz increased the oxidation state of mitochondria in neurons and reduced glial LD accumulation, whereas knockdown of GLaz decreased glial mitochondrial oxidation, arguing in favor of lipid transfer from neurons to glia underlying this phenomenon. This mechanism thus reproduces, in physiological conditions, the

mechanism of lipid transfer from neurons to glia that was previously characterized as alleviating oxidative damage to neurons in pathologically defective mitochondria (discussed in the previous section). In the context of sleep need, this lipid transfer allows exporting oxidative damage caused by elevated neuronal activity during wakefulness from neurons to glia (Haynes et al. 2024). Remarkably, fatty acid β-oxidation is required in both cortex and ensheathing glia for normal sleep, whereas sleep promotes glial lipid catabolism and clearance of damaged mitochondria through selective mitophagy. These findings thus reveal a sleep-regulated metabolic cycle between neurons and glia involving lipid transfer and oxidation, whereby an essential function of sleep is to clear oxidized lipids and damaged mitochondria in order to maintain the efficiency of neuronal energy production during wake states (Haynes et al. 2024).

Whether the sleep-promoting role of ecdysone is related to the triggering of LD catabolism and the wake–sleep transition remains to be investigated. Nevertheless, these two recent studies firmly link glial lipid oxidation, in particular in cortex glia, to sleep. As previously mentioned, neuronal fatty acid synthesis and lipid transfer from neurons to glia are enabled by reciprocal lactate exchange (Liu et al. 2017). In mammals, brain lactate levels are known to be higher during the wake state than during sleep (Shram et al. 2002; Naylor et al. 2012), which is assumed to rely on extracellular lactate clearance by the glymphatic system during sleep (Lundgaard et al. 2017; Benveniste et al. 2019). Intriguingly, it was shown that *Drosophila* Ldh exhibits increased circadian rhythmic expression as fly age, but also that oxidative stress induces rhythmic Ldh expression in young flies (Kuintzle et al. 2017). These findings invite us to explore the possibility that, like in mammals, the fly brain could show oscillations in lactate levels with sleep/wake cycles, which might occur in coordination with the building need of neuronal lipid transfer to glia during wakefulness (Haynes et al. 2024) and become more prominent as the burden of oxidative stress increases during the course of aging. Finally, because FA oxidation occurs in glia during sleep, it would be interesting to investigate if the residual neuronal activity during sleep is fueled by glia-derived KBs, as in a starvation state, rather than by glucose metabolism.

Conclusion

Studies on the role of glial cells in the adult *Drosophila* brain have been extremely fruitful in recent years, revealing a remarkable diversity in the way that glial metabolic pathways are connected with neuronal physiology to influence behavior. Not all of the effects reviewed here are linked with certainty to the MB region, in particular those concerning brain-wide phenomena such as sleep. However, learning and memory studies have made it clear that the properties of memory encoding are deterministically linked to the spatial, temporal, and biochemical patterns of glial metabolic activation. Thus, in addition to advances in the description and understanding of MB neuronal networks that have characterized MB research in the past 20 years, glial cells have emerged as a critical component to be integrated into future research. It should be noted that studies so far have primarily focused on the glia–KC interaction, whereas the glia interaction with KC partners (i.e., afferent dopaminergic neurons and MB output neurons) is likely to yield important discoveries as well. This perspective was pioneered in a recent study (Miyashita et al. 2023) demonstrating that glutamate release from ensheathing glia acts on KCs and upstream dopamine neurons during aversive associative learning to signal the delivery of a punishing stimulus. Significantly, this report of a purely signaling role for glial cells (and not a metabolic role) has uncovered an exciting new area of research into the role of glial interventions in MB-related behavior.

Competing interest statement

The authors declare no conflict of interest.

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